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Daily and Seasonal Rhythms of Melatonin, Cortisol, Leptin, Free Fatty Acids and Glycerol in Goats

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original papers, which are referred to in the text by their Roman numerals:

I Alila-Johansson, A., Eriksson, L., Soveri, T., Laakso, M-L., 2001. Seasonal variation in endogenous serum melatonin profiles in goats: a difference between spring and fall? J. Biol. Rhythms 16, 254-263.

II Alila-Johansson, A., Eriksson, L., Soveri, T., Laakso, M-L., 2003. Serum cortisol levels in goats exhibit seasonal but not daily rhythmicity. Chronobiol. Int. 20, 65-79.

III Alila-Johansson, A., Eriksson, L., Soveri, T., Laakso, M-L., 2004. Daily and annual variations of free fatty acid, glycerol and leptin plasma concentrations in goats (*Capra hircus*) under different photoperiods. Comp. Biochem. Physiol. A138, 119-131.

IV Alila-Johansson, A., Eriksson, L., Soveri, T., Laakso, M-L., 2006. The daily rhythms of melatonin and free fatty acids in goats under varying photoperiods and constant darkness. Chronobiol. Int. 23, 565-581.

ABBREVIATIONS

ACh	acetylcholine
ACTH	adrenocorticotropic hormone (corticotropin)
ANOVA	analysis of variance
AUC	area under curve
AVP	arginine vasopressin
CORT	cortisol
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
DD	continuous darkness
DMH	dorsomedial hypothalamic nucleus
E	evening oscillator
EEG	electroencephalogram
FAA	food anticipatory activity
FEO	food-entrainable oscillator
FFA	free fatty acid (NEFA non-esterified fatty acid)
GABA	γ-aminobutyric acid
GLY	glycerol
HPA	hypothalamic-pituitary-adrenal
IGL	thalamic intergeniculate leaflet
IMLC	intermediolateral cell column
LD	light/dark
LEP	leptin
M	morning oscillator
MEL	melatonin
MEL-R	melatonin receptor
NA	noradrenaline
PROG	progesterone
PVN	paraventricular nucleus of hypothalamus
SCG	superior cervical ganglion
SCN	suprachiasmatic nucleus
SD	standard deviation
SEM	standard error of the mean
VHM	ventromedial hypothalamus
VIP	vasoactive intestinal polypeptide

SUMMARY

Daily and annual variations in melatonin, cortisol and leptin levels and in lipid metabolism of Finnish female landrace goats were investigated. The animals were kept under artificial lighting conditions that approximately simulated annual changes in natural photoperiods. Ambient temperature and feeding regime were kept constant. Study variables were obtained from blood samples collected in different seasons over 24-h periods at 2-h intervals, first in light/dark (LD) conditions and then in continuous darkness (DD). The experiments were performed in Helsinki, Finland (60 ˚N).

The results demonstrate that the melatonin signal carries reliable information about seasonal changes in the photoperiod in goats. The duration of melatonin secretion in LD closely follows the length of the dark phase, except in winter, when the duration is significantly shorter. In DD, the goats display two types of endogenous melatonin patterns: a "winter pattern" in winter, early spring, early fall and late fall, and a "summer pattern" in late spring and summer. In equal habitual LD conditions in late spring and early fall (LD 14/10), the endogenous melatonin rhythms after one day in DD were found to be somewhat different: the pattern in late spring resembled that in summer, and the pattern in early fall that in winter. But after 3 days in DD, the patterns under equal photoperiods in spring and fall were not different. Endogenous melatonin secretion can be modulated by circannual clock mechanisms and/or by long-term photoperiodic history.

The measurement of circadian cortisol levels failed to demonstrate any clear endogenous daily rhythm in serum cortisol levels. The small variations found were probably related to external conditions, the feeding schedule being the most likely contributing factor. On the other hand, the duration of photoperiod and the direction of its change over a year significantly influenced the overall cortisol levels.

The measurements of lipid metabolism demonstrated daily and seasonal variations in the concentrations of plasma free fatty acids (FFAs) and glycerol, whereas no such variation was detected in serum leptin levels. The daily rhythm of glycerol was associated with concentrate meal times throughout the year, whereas the rhythm of FFA was related to the daily LD fluctuations. In DD conditions, FFA and glycerol rhythms were unstable. The dependence of the FFA rhythm on daily lighting conditions is a novel finding. This dependence is suggested to be generated by an endogenous oscillator, primarily adjusted by dawn.

The study demonstrates an association between the daily and annual rhythms of melatonin and FFA. In LD conditions, both melatonin and

FFA displayed constant rhythmicity. Melatonin secretion was entrained by lights-off and lights-on times, except in winter. The FFA levels were low at night, with a transient peak around lights-on. There was an overall parallelism between the two rhythms, with one significant exception: in winter in LD conditions, the morning rise of FFA levels coincided with lights-on and not with the declining phase of melatonin, whereas in DD conditions, the FFA peak advanced several hours and coincided with the declining phase of melatonin. This result and other rhythm characteristics indicate that the daily rhythm of blood FFA levels is regulated by an intrinsic clock, which in turn is controlled by light and especially by dawn. The close relationships between the daily variations of melatonin and FFA levels result from both variables being controlled by light-dependent mechanisms. The results do not rule out a possible effect of melatonin on the daily FFA profiles, but they show that melatonin secretion alone could not completely explain the daily FFA profiles.

Lighting conditions have earlier been shown to participate in the regulation of annual variations in lipid metabolism in several animal species. This study demonstrates that lighting conditions also have an impact on daily variations in lipid metabolism, although the mechanisms remain unknown. Further investigations are needed to clarify these mechanisms both at the cellular level and at the whole-organism level.

INTRODUCTION

Most of life on earth has adapted to the daily change of light intensity; organisms organize their activities in ca. 24-h cycles determined by sunrise and sunset. This circadian rhythm affects animals´ sleeping and feeding patterns, brain wave activity, hormone production and other biological activities related to the daily cycle. In the retina, light is transformed into nerve impulses that are conveyed to the hypothalamic nuclei of the central nervous system. Hypothalamic suprachiasmatic nuclei (SCNs) are the principal generators of circadian rhythms and a part of the entrainment system that synchronizes the animal with its environment, especially with lighting conditions (Klein et al., 1991). The daily alternation of light and dark is the most salient regulatory factor in production of the pineal hormone melatonin. The daily pattern of melatonin secretion also depends on the function of the suprachiasmatic circadian clock since the prevailing illumination is mediated by the retinohypothalamic connections via the SCN to the pineal gland (Moore and Klein, 1974).

Changes in long-term lighting conditions occurring during the year result in metabolic and behavioural changes. For instance, the reproductive cycles with concurrent hormonal changes are entrained in many mammalian species by annual lighting conditions (Karsch et al., 1984). Evidence suggest that the SCN-pineal complex is a probable component in the mechanism entraining annual rhythms (Zucker et al., 1991; Scott et al., 1995). Melatonin regulates seasonal breeding by synchronizing the reproductive functions with the duration of high-rate melatonin secretion. It serves the reproductive system as a messenger of night length (Karsch et al., 1991; Bartness et al., 1993). In nature, another requirement of seasonal alterations in metabolism and behaviour is associated with variation of environmental conditions, for instance, with the availability of food.

In addition to the melatonin pattern, the daily variation of glucocorticoid levels in blood is a classical example of circadian rhythms in humans (Bliss et al., 1953; Orth and Island, 1969), rats (*Rattus norvegicus,* Guillemin et al., 1959; Moore and Eichler, 1972), pigs (*Sus scrofa,* Whipp et al., 1970; Andersson et al., 2000), horses (*Equus przewalski,* James et al., 1970; Irvine and Alexander, 1994), rhesus monkey (*Macaca mulatta,* Perlow et al., 1981), Syrian hamsters (*Mesocricetus auratus,* de Souza and Meier, 1987) and red-backed voles (*Clethrionomys gapperi,* Kramer and Sothern, 2001). This phenomenon has been described in many mammalian species, but findings in ruminants, such as in cattle (*Bos taurus,* MacAdam and Eberhart, 1972; Wagner and Oxenreider, 1972; Abilay and Johnson, 1973;

Paape et al., 1974; Hudson et al., 1975; Fulkerson et al., 1980; Thun et al., 1981; Lefcourt et al., 1993), sheep (*Ovis aries,* McNatty et al., 1972; Basset, 1974; Barrell and Lapwood, 1978; Fulkerson and Tang, 1979; Kennaway et al., 1981; Lincoln et al., 1982; McMillen et al., 1987; Simonetta et al., 1991), white-tailed deer (*Odocoileus virginianus,* Bubenik et al., 1977, 1983) and Eld´s deer (*Cervus eldi thamin,* Monfort et al., 1993; Ingram et al., 1999), have been absent, scant or inconsistent. These marginal or discrepant results in ruminants might be explained by the central role of glucocorticoids in the regulation of several factors of metabolism, including feeding. Due to the special digestive system of ruminants, nutrients are absorbed into the blood rather evenly throughout the 24-h period. Daily variations in metabolites and metabolic hormones are therefore expected to be small in ruminants with free access to food.

The impact of lighting conditions on the regulation of lipid metabolism has been documented in several species of mammals (for reviews, see Clarke, 2001; Bartness et al., 2002; Morgan et al., 2003), and the pineal hormone melatonin seems to be a universal mediator of photoperiodic information in this regulation (Lincoln et al., 2003). There is, however, less knowledge about the role of melatonin in the regulation of energy metabolism on a daily basis.

This study was conducted to gain more information about daily and annual variations and possible interactions of the blood levels of melatonin, cortisol, leptin, free fatty acids and glycerol in goats kept in artificial lighting simulating the annual changes of the natural photoperiod in Helsinki, Finland (60°N). To characterize possible endogenous rhythms, the blood levels of these substances were also determined in continuous darkness.

REVIEW OF THE LITERATURE

DAILY RHYTHMS

The physiological processes of organisms are regulated by a circadian rhythm, the length of which is approximately 24 h. This rhythm was first described in the movement of plant leaves by the French scientist Jean-Jacques d'Ortous de Marian (de Marian, 1729; referred by Meijer and Rietveld, 1989). The circadian system is regulated by the wavelength, intensity, timing and duration of the light stimulus (Cardinali et al., 1972; Brainard et al., 1983, 1986; Takahashi et al., 1984). These endogenous biological rhythms allow us to anticipate periodic changes in the environment and are thus important for adaptive behaviour.

Light-entrained circadian rhythms

Although evolvement of the circadian rhythm is related to the light/dark cycle of the solar day, it also persists in constant conditions (e.g. constant light). The rhythm period can be reset by exposure to a light or dark pulse, and if there is a change in the lighting conditions, the animal can gradually adjust to the new pattern provided it does not deviate too much from the species norm.

Animals that are kept in total darkness for a long period of time start to display a "free-running" rhythm (Redman et al., 1983; Thomas and Armstrong, 1988). The sleep cycle of diurnal animals moves forward approximately one hour a day; their free-running rhythms are about 25 h. In nocturnal animals, by contrast, the free-running rhythm is about 23 h. Even in total darkness, unless the environment is shielded from all external cues, the free-running rhythm is influenced by events occurring regularly on a daily basis. Continuous light treatment induces suppression of circadian rhythmicity of locomotor activity (Homna and Hiroshige, 1978; Chesworth et al., 1987) in rats. Several other circadian rhythms in rats (e.g. behavioural, temperature and some humoral rhythms) may persist for several weeks depending on the intensity of light (Homna and Hiroshige, 1978; Eastman and Rechtschaffen, 1983; Deprés-Brummer et al., 1995).

The environmental cues that entrain the circadian rhythm are called Zeitgebers or circadian synchronizers. Several environmental and behavioural stimuli act as circadian synchronizers. These include water and food intake, motor activity, sleep-wake rhythm, corticosterone release, activity

of pineal N-acetyltranferase enzyme and body temperature (reviewed by Rusak and Zucker, 1979). The most important synchronizing trigger of circadian rhythmicity is, however, environmental light/dark (LD) cyclicity. In the absence of external cues, the rhythm may become out of phase with, for instance, the ultradian rhythm of digestion. Biological functions, such as hormone production, cell regeneration and brain activation as measured by an electroencephalogram (EEG), and overall behavioural patterns (sleeping, eating) are linked to the circadian cycle.

In mammals, the master circadian pacemaker, the biological clock that provides endogenous circadian cycles, is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Destruction of the SCN completely abolishes the normal sleep/wake rhythm. Information about day length travels from the SCN to the pineal gland. In response to this information, the pineal gland secretes the hormone melatonin. The secretion reaches its peak at night and wanes during the day (Zucker et al., 1983). The findings of recent studies indicate that the SCN can also spread its message directly to peripheral organs and tissues through the autonomic nervous system (Bartness et al., 2001; la Fleur, 2003; Buijs et al., 2006).

Feeding-entrained circadian rhythms

A feeding-entrained circadian system, which seems to be independent of the light-dark fluctuations of the solar day, has been described in animals (Mistlberger, 1994; Stephan, 2002). Temporal restriction of feeding (RF) can phase-shift behavioural and physiological circadian rhythms in mammals. These changes in biological rhythms are postulated to be caused by a food-entrainable oscillator (FEO) that is independent of the SCN (Mieda et al., 2006). When food availability is restricted to a single period scheduled at a fixed time of the day, mice (*Mus musculus*) adapt to this condition within a few days by feeding during the period of food availability and increasing food-seeking activity in the preceding hours (food anticipatory activity, FAA; Hastings et al., 2003; Lowrey and Takahashi, 2004). Phase advances of circadian rhythms have also been observed in gene expression. This happens, for instance, in the liver, kidney, heart, pancreas and some brain structures, uncoupling them from the control of the SCN, whose entrainment to light remains intact (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Wakamatsu et al., 2001; Mendoza, 2006). Feeding-fasting signals might be involved in the entrainment of the peripheral circadian oscillators (Damiola et al., 2000; Stokkan et al., 2001).

Existence of the FEO has not been unequivocally established. Some studies suggest that the dorsomedial hypothalamic nucleus (DMH) is a key structure for FEO expression (Gooley et al., 2006, Mieda et al., 2006). The results of a study in rats with electrolytic DMH lesions does not, however, support this hypothesis (Landry et al., 2006). Neither is the circadian mechanism of FEO at the molecular level yet clear (Mendoza, 2006). Al-

though evidence supporting the existence of this feeding-entrained circadian system has been obtained only during restriction of feeding (RF), it is likely that if such a system exists it would also participate in the regulation of body rhythms in everyday conditions.

ANNUAL RHYTHMS

The gradually changing annual lighting conditions have a strong impact on the behaviour and physiology of most mammalian species (Hastings et al., 1985). The timing of annual reproductive cycles associated with hormonal changes is an important factor in the successful production of offspring (Karsch et al., 1984). Besides lighting, other seasonal alterations affect the metabolism of animals. These include variation in temperature, humidity and availability of food.

Accumulating evidence indicates that in mammals the SCN and the pineal gland are the main structures regulating annual cycles (Hastings, 2001; Schwartz et al., 2001; Zucker, 2001). This timing system in the brain regulates such seasonal cycles as sexual behaviour, energy metabolism, food intake and hibernation. In most species, the photoperiod appears to be the strongest synchronizer of seasonal functions. For example, in Djungarian hamsters (*Phodopus sungorus*), short days induce reproductive inhibition, inactivity and weight increase, while animals kept in long days do not display these changes. In sheep, reversal of the annual photoperiodic cycle causes the breeding season to phase shift by 6 months; reduction of its period to 6 months triggers two periods of reproductive activity every year (see Malpaux et al., 1993, 2001).

The following findings support the view that the hypothalamic circadian clock in the SCN is the site of integration of annual changes in photoperiod (for review, see Goldman, 2001; Schwartz et al., 2001): a circadian reading of the photoperiod appears necessary (Maywood et al., 1990); FOS reactivity in the SCN following a light stimulus depends on the photoperiod history (Sumova et al., 1995; Vuillez et al., 1996); clock gene expression in the SCN displays photoperiodic variations (Messager et al., 1999, 2000, 2001; Nuesslein-Hildesheim et al., 2000); and the daily profile of arginine vasopressin (VP) messenger ribonucleic acid (mRNA) differs in long and short photoperiods (Jac et al., 2000). There is also evidence that the thalamic intergeniculate leaflet (IGL), a relay between the retina and SCN, is involved in photoperiod integration (Menet et al., 2001).

Some photoperiodic species, e.g. ground squirrels (*Spermophilus parryii*), exhibit endogenous circannual rhythms when they are kept under seasonally constant conditions (photoperiod and temperature) for long periods of time (Lee and Zucker, 1991; Gorman et al., 2001; Zucker, 2001). However, the anatomical substrate of the circannual timing system has not been confirmed in any mammalian species.

The SCN generates the circadian oscillators. Pittendrigh and Daan (1976) suggested that there is a morning oscillator (M) adjusted by dawn and an evening oscillator (E) adjusted by dusk in the mammalian circadian system. The phase relationship between M and E reflects the day length to which the animal has been exposed. The oscillators control the pineal gland, therefore being able to define not only the time of day but also the time of year (Schwartz et al., 2001).

The hypothesis of Daan and colleagues (2001) suggests that the circadian pacemaker in the mammalian SCN consists of a double complex of circadian genes (Per1/Cry1 and Per2/Cry2), which is able to maintain the endogenous rhythmicity, thus forming oscillators. These two oscillators are speculated to have slightly different temporal dynamics and light responses. The Per1/Cry1 (or M) oscillator is apparently accelerated by light and decelerated by dark, and the Per2/Cry2 (or E) oscillator is decelerated by light and accelerated by dark. Changes in the activity of these oscillators may have an influence on the adaptation of the endogenous behavioural programme to day length.

PINEAL GLAND AND MELATONIN

The pineal gland (glandula pinealis, epiphysis cerebri) is a small structure located in most mammals between the habenular and posterior commissures. Identification of the pineal gland as a distinct cerebral organ can be traced back to the 3rd and 4th centuries BC (Kappers, 1960; Hoffman and Reiter, 1965). At the end of the 19th century, Ahlborn and Rabl-Ruckhardt, then Graaf, Korschelt and Spencer, described the anatomy, histology, innervation and embryology of the mammalian pineal gland and noted its resemblance to the epiphysis organ of lower vertebrates (reviewed by Simonneaux and Ribelayaga, 2003). In 1905, Studnicka established that phylogenetically the pineal gland is derived from a photoreceptor organ, but its function remained unknown (reviewed by Simonneaux and Ribelayaga, 2003). Modern bioassay techniques enabled the discovery of an active pineal extract capable of lightening the colour of frog skin (McCord and Allen, 1917); this was followed by the isolation of the pineal hormone melatonin in 1958 (Lerner et al., 1958, 1959). Fluorescent techniques allowed the measurement of melatonin and serotonin concentrations, which led to the discovery of the large circadian variations in their levels (Quay, 1963, 1964).

Innervation of the pineal gland

Peripheral sympathetic and parasympathetic fibres and those originating from the central nervous system innervate the mammalian pineal gland. The pineal gland differs from other brain structures inasmuch as it receives

relatively scarce afferent innervation from the brain itself. The most important afferents are postganglionic sympathetic fibres originating from the superior cervical ganglia (SCG) and forming the bilateral *nervi conarii*, which enter the pineal posteriorly (Kappers, 1979). The neurons of this noradrenergic pathway receive regulatory input from the suprachiasmatic nucleus of the hypothalamus, which receives direct input from retinal ganglion cells (Kappers, 1960, 1979) via the monosynaptic retinohypothalamic tract. The transmission of this tract is probably modulated both pre- and post-synaptically by neuropeptide Y (NPY) in many mammals (Simonneaux et al., 1994; Mikkelsen et al., 2000).

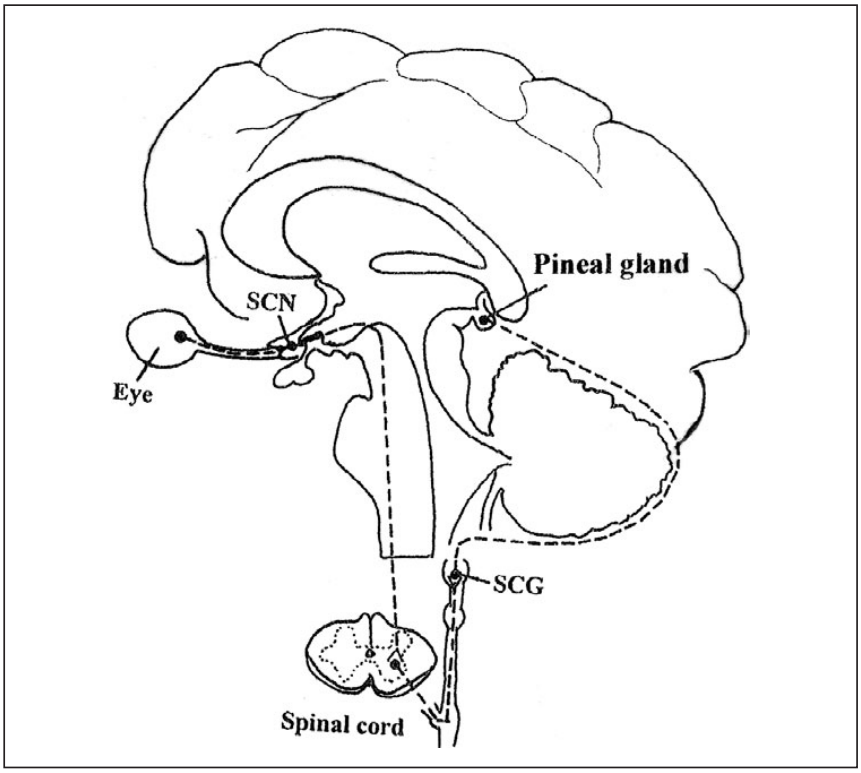

Figure 1. REGULATION PATHWAY OF PINEAL MELATONIN
Sagittal view of the mammalian brain showing the pineal gland and its innervation. Retinohypothalamic fibres synapse in the suprachiasmatic nuclei (SCN), and connections from the SCNs to the intermediolateral grey column in the spinal cord exist. Preganglionic neurons pass from the spinal cord to the superior cervical ganglion (SCG), and the postganglionic neurons project from this ganglion to the pineal gland in the *nervi conarii* (modified from Andersson, 1978 and Ganong, 1997).

Anteriorly, the pineal gland receives afferents that travel through the commissural peduncles, possibly originating from the hypothalamus (Vollrath, 1984). A third pathway, the ventro-lateral pineal tract, has also been described (Sparks, 1998). The myelinated fibres of this tract originate from

the pretectal region, posterior and lateral to the posterior commissure. Central nerve fibres originating from the hypothalamic, limbic forebrain and visual structures have been shown to innervate the pineal gland in non-human mammals (Kappers, 1960). As to the parasympathetic innervation of the pineal gland, fibres containing the primary neurotransmitter of parasympathetic neurons, acetylcholine (Ach), have been described in some mammalian species, including the cow and rat (Phansuwan-Pujito et al., 1991; Korf et al., 1996; Weihe et al., 1996). There is also evidence of parasympathetic fibres arising from the mammalian pterygopalatine ganglia and containing vasoactive intestinal peptide (VIP) and other neuropeptides (Møller, 1992).

Besides NPY, which is located in the sympathetic fibres, and VIP, many other peptides have been found in nerve fibres terminating in perivascular and intraparenchymal areas in mammals. These include substance P, vasopressin, oxytocin and luteinizing hormone-releasing hormone (Barry, 1979; Ronnekleiv, 1988). Although Substance P fibres might arise from the habenula (Larsen et al., 1991), the origin of these peptidergic fibres remain obscure.

Synthesis and metabolism of melatonin

Melatonin (5-methoxy-N-acetyltryptamine) is a small (molecular weight 232.3) indoleamine secreted rhythmically, with increased synthesis at night. Tryptophan acts as a precursor in the biosynthesis of melatonin. Pinealocytes hydroxylate and decarboxylate tryptophan to serotonin, which is then acetylated to N-acetyl-serotonin by the rate-limiting enzyme N-acetyl transferase (NAT). This is methylated by hydroxyindole-O-methyltransferase (HIOMT) to melatonin (Reiter, 1991). After its biosynthesis, the highly lipophilic melatonin is released into capillaries, where most of it binds to albumin (Cardinali et al., 1972). Melatonin is metabolized by hydroxylation and conjugation with sulphate or glucuronic acid, mainly in the liver but also in the kidney, finally being excreted into urine as 6-sulphatoxymelatonin. Functional disorders of these organs have been shown to affect the elimination rate (Lane and Moss, 1985; Viljoen et al., 1992; Kunz et al., 1999). The half-life of melatonin in blood after intravenous administration is about 30 min (Mallo et al., 1990).

In addition to blood, urine and saliva, melatonin has been found in the cerebrospinal fluid (CSF), at a concentration much higher than in blood, and in the anterior chamber of the eye (Martin et al., 1992). Melatonin is also found in semen, amniotic fluid, urine and breast milk (Cagnacci, 1996). Melatonin in plasma, CSF, saliva and urine is eliminated by pinealectomy, indicating that melatonin is mainly synthesized in the pineal gland (Nelson and Drazen, 1999). There is, however, evidence that melatonin is also synthesized elsewhere; the other sites include, in humans, the retina, gut and bone marrow (Cagnacci, 1996; Conti et al., 2000). This

means that, besides a central regulatory function, melatonin also has a localized action (Fjaerli et al., 1999).

Melatonin receptors

In humans, there are two types of melatonin receptors (Mel_{1a} and Mel_{1b}) with different binding affinity and chromosomal localization (Reppert et al., 1995). Melatonin receptors have been found in the SCN of the hypothalamus, which controls the rhythmic production of melatonin by the pineal gland (Reppert et al., 1988; Weaver and Reppert, 1996). They are also located in the cerebellum (Al-Ghoul et al., 1998), retinal rods, horizontal amacrine and ganglion cells (Reppert et al., 1995; Scher et al., 2002). Besides the CNS, human melatonin receptors have been found in lymphocytes (Lopez-Gonzalez et al., 1992; Konachkieva et al., 1995), prostate epithelial cells (Zisapel et al., 1998), granulose cells of preovulatory follicles (Yie et al., 1995), spermatozoa (van Vuuren et al., 1992), the mucosa layer of the colon (Poon et al., 1996) and blood platelets (Vacas et al., 1992). Melatonin molecules exert systemic effects also at the basic cellular level, in the absence of receptors (Benitez-King, 1993; Fjaerli et al., 1999).

DAILY RHYTHM OF MELATONIN

The daily alternation of light and dark is the most important regulatory element in the synthesis of pineal melatonin. In all mammals studied to date, whether they exhibit nocturnal or diurnal activity, melatonin levels are higher at night than during the day. The melatonin level starts to rise during the evening and is at its highest in the middle of the night and starts to decrease during the morning. The daily rhythm of melatonin is considered to be a very reliable phase marker used by the endogenous timing system. In the absence of the LD cycle, melatonin rhythms begin to free-run with a period slightly different from 24 h (Aschoff, 1965). In rats, Syrian hamster and Siberian hamster (*Phodopus sungorus*) with free-running circadian rhythms, pharmacological doses of exogenous melatonin are capable of synchronizing the circadian rhythms of locomotor activity and melatonin synthesis (for review, see Redman et al., 1983; Humlova and Illnerova, 1990; Kirsch et al., 1993; Grosse and Davis, 1998; Pitrosky et al., 1999; Schuhler et al., 2002). The circadian rhythm of melatonin is intrinsic and it persists in a non-periodic environment, i.e., in continuous darkness or very dim light. Lesioning the SCN abolishes all pineal melatonin rhythmicity (Klein and Moore, 1979), demonstrating that input from the main endogenous circadian pacemaker, located in the SCN, is essential for the circadian rhythm of melatonin and its synchronization with the external LD cycle (entrainment).

Light, in addition to entraining the circadian rhythm, can directly suppress nocturnal melatonin levels. A significant suppression in synthesis is dependent on the dose of light. In humans, a decrease in nocturnal melatonin levels has been produced by relatively low lighting intensities, such as those in normal indoor lighting (Lewy et al., 1980; McIntyre et al., 1989; Laakso et al., 1991, 1994; Brainard et al., 2001). Light is also able to phase-shift the nocturnal melatonin rhythm.

In endogenous circadian rhythms of mammals, melatonin has been shown to act as a synchronizer (Armstrong, 1989). The synchronizing effect occurs at a particular circadian time, differing according to species (e.g., at the beginning of the active period in rats). Exogenous melatonin, applied directly into the SCN by reverse microdialysis, not only phase-advances the endogenous melatonin peak but also increases its peak (Bothorel et al., 2002). Various *in vitro* studies have also demonstrated a local effect of melatonin on SCN metabolism, electrical activity and circadian rhythmicity (Cassone et al., 1988; Stehle et al., 1989; McArthur et al., 1991). Small doses of exogenous melatonin are known to induce a phase-shifting effect in rats (Warren et al., 1993) and humans (Lewy et al., 2005). Melatonin may exert its synchronizing properties indirectly on clock inputs and outputs or directly on the clock via melatonin receptors (MEL-R was identified in vasopressin-containing SCN neurons; Song et al., 1999) or other binding sites (for review, see Pévet et al., 2002). This property of melatonin is used, along with several circadian signals, between the mother and the foetus to entrain the circadian clock of offspring (Reppert et al., 1979; Reppert and Weaver, 1991).

In humans, this "chronobiotic" function of melatonin helps to re-synchronize the rhythms of individuals with disrupted circadian rhythms. A disrupted rhythm can, for example, be due to "delayed sleep phase" syndrome, jet-lag, night shift work or blindness (Arendt et al., 1984, 1987, 1988, 1997; Lockley et al., 2000; Takahashi et al., 2000).

ANNUAL RHYTHM OF MELATONIN

Accumulating evidence indicates that in mammals the SCN and the pineal gland are the principal neural structures involved in the regulation of annual cycles (for review, see Goldman, 2001; Schwartz et al., 2001; Zucker, 2001). The pineal gland is a major structure in the endocrine system allowing mammals to respond to annual changes in the photoperiod by adaptive alterations of their physiological state. The cyclicity of reproductive behaviour of most mammals is an example of adaptive behaviour that is entrained by alterations of day length during the year. The pineal gland and its melatonin rhythm are essential triggers of this cyclicity of reproduction;

numerous studies have demonstrated that the pineal gland is a neuroendocrine transducer receiving photoperiodic information from the retina and circadian SCN oscillator and transmitting this to the reproductive system via a particular dynamic pattern of melatonin secretion (for review, see Hoffmann, 1979; Reiter, 1980; Goldman and Darrow, 1983; Bittman, 1984; Tamarkin et al., 1985; Pévet, 1988; Goldman, 2001).

Periodic synthesis and secretion of melatonin by the pineal gland are generated by oscillators in the SCN that is connected to the pineal gland via a complex multisynaptic pathway (Kalsbeek et al., 1993; Buijs, 1996; Teclemariam-Mesbah et al., 1999; Kalsbeek and Buijs, 2002). The findings that disruption of any portion of this pathway abolishes melatonin rhythmicity suggest that the mammalian pineal gland receives major input from the SCN, and thus, its endocrine activity is neurally controlled. These findings also indicate that, besides being a circadian pacemaker, the SCN has the capability of communicating seasonal timing. In fact, several physiological and behavioural processes have been found to be regulated by photoperiod-dependent changes in melatonin secretion (Cassone, 1990; Lee and Zucker, 1991; Arendt, 1995; Pévet et al., 1996; Wehr, 1997; Goldman, 2001; Gorman et al., 2001).

The duration of melatonin secretion is inversely related to day length. It is therefore possible that the melatonin signal encodes information about gradual changes in day length during the year. Several hypotheses have been proposed concerning which parameters of the melatonin secretion pattern (duration, amplitude, phase or total quantity) convey the photoperiodic message to target structures. The hypotheses have been based on the analyses of endogenous melatonin patterns in different experimental conditions and on experiments investigating the effects of acute injections or chronic infusions of exogenous melatonin (for review, see Carter and Goldman, 1983; Pitrosky et al., 1991; Bartness et al., 1993). Studies of pinealectomized Siberian and Syrian hamsters and sheep administered daily infusions of melatonin indicate that photoperiodic information is indeed encoded in the melatonin signal (for review, see Bartness et al., 1993). In both sheep breeding during short days and hamsters breeding during long days, short-duration melatonin infusions resulted in long-day responses typical of the species, whereas long-duration infusions elicited responses associated with short days. These results suggest that the signal about day length is encoded in the duration of nocturnal melatonin secretion. In other words, the melatonin pattern serves as a humoral signal conveing day length information (Schwartz et al., 2001; Stehle et al., 2001). Furthermore, observations of the melatonin secretion pattern in various species kept in different photoperiodic conditions have shown that the duration of the nocturnal melatonin peak is positively related to length of the night (sheep: Rollag and Niswender, 1976; Karsch et al., 1988; rat: Illnerova and Vanecek, 1980; Siberian hamster: Illnerova et al., 1984; Ribelayga et al.,

2000; Syrian hamster: Skene et al., 1987; Maywood et al., 1993; Miguez et al., 1995; European hamster (*Cricetus cricetus*, Vivien-Roels et al., 1992).

Moreover, the amplitude of the nocturnal peak of melatonin seems to be an important factor in photoperiodic transmission (for review, see Vivien-Roels, 1999). There are several studies showing that the amplitude reflects photoperiodic variation. These studies include investigations in the pig (*Sus scrofa,* McConnell and Ellendorf, 1987; Taste et al., 2001), mule (*Equus asinus x Equus caballus,* Cozzi et al., 1991), Siberian hamster (Lerchl and Schlatt, 1992; Steinlechner et al., 1995; Miguez et al., 1996; Ribelayga et al., 2000), European hamster (Vivien-Roels et al., 1992, 1997) and horse (Guérin et al., 1995).

The early experiments on the effects of melatonin showed that an acute injection of melatonin at the end of the day or the beginning of night to hamsters kept in a long photoperiod induced gonadal regression, while a similar injection at the end of night or the beginning of day had no effect. This finding indicates that there is a phase of sensitivity during which the injection of melatonin has to be administered in order to have a physiological effect (for review, see Tamarkin et al., 1976; Reiter, 1987).

CORTISOL

Cortisol is a glucocorticoid hormone that is involved in the response to stress; it increases blood pressure and blood sugar levels and suppresses the immune system. Synthetic cortisol, also known as hydrocortisone, is used as a drug mainly to fight allergies and inflammation (e.g. Grego, 2002).

The synthesis of cortisol in the cortex of the adrenal gland is stimulated by the anterior lobe of the pituitary gland with adrenocorticotropic hormone (ACTH); the production of ACTH is in turn stimulated by corticotropin-releasing hormone (CRH), released by the hypothalamus.

Cortisol also inhibits the secretion of CRH, resulting in feedback inhibition of ACTH secretion. Some evidence indicates that this normal feedback system may break down when animals are exposed to chronic stress (e.g. Grego, 2002).

DAILY RHYTHM OF CORTISOL

The daily variation of glucocorticoid levels in blood is a classical example of circadian rhythms. It has been demonstrated in several mammalian species including humans (Bliss et al., 1953; Orth and Island, 1969), rats (Guillemin et al., 1959; Moore and Eichler, 1972), pigs (Whipp et al., 1970; Andersson et al., 2000), horses (James et al., 1970; Irvine and Alexander, 1994), rhesus monkey (Perlow et al., 1981), hamsters (de Souza et al., 1987)

and red-backed voles (Kramer and Sothern, 2001). The concentrations are at their lowest during rest, and there is a rapid rise just before the active period begins, in both day-active and nocturnal species. In the rat, the secretion of glucocorticoid hormones has been shown to be under the control of the main body clock, the SCN (Moore and Eichler, 1972). However, there is evidence that the rhythm can be maintained also by other mechanisms; in primates with lesions in the suprachiasmatic region, the daily cortisol rhythm does not disappear (Reppert et al., 1981).

The morning rise of cortisol in day-active animals is endogenous. In humans, the rise can be enhanced by bright light after awakening (Scheer Buijs, 1999) and also by sleep deprivation (Leproult et al., 2001). In pigs, exposure to supplementary artificial light in the morning after sunrise increased the cortisol level (Andersson et al., 2000), and in male Creole goats (*Capra hircus*), an abrupt exposure to sunlight in the middle of the day resulted in enhanced cortisol concentrations (Sergent et al., 1985). The intensity of daytime lighting as such has been found not to affect cortisol levels in pigs (Griffith and Minton, 1992) or in bulls (Leining et al., 1980).

The results concerning the patterns of cortisol levels in ruminants are inconsistent; the levels have either been observed to fluctuate episodically or peaks and troughs have been found at varying times of the day depending on the conditions. The absence of circadian variation of cortisol levels has been a common finding in studies with sheep (McNatty et al., 1972; Basset et al., 1974; Barrell and Lapwood, 1978; Fulkerson and Tang, 1979; Kennaway et al., 1981; Lincoln et al., 1982; McMillen et al., 1987; Simonetta et al., 1991), white-tailed deer (Bubenik et al., 1977, 1983), Eld´s deer (Monfort et al., 1993; Ingram et al., 1999) and cattle (MacAdam and Eberhart, 1972; Wagner et al., 1972; Abilay et al., 1973; Paape et al., 1974; Hudson et al., 1975; Fulkerson et al., 1980; Lefcourt et al., 1993).

However, when samples are collected frequently or values are pooled, significant or marginal fluctuations of cortisol levels have been observed in ruminants. Serum cortisol levels have been reported to increase during the night or in the morning in goats (Kokkonen et al., 2001), sheep (McNatty et al., 1972; Holley et al., 1975; Kennaway et al., 1981; McMillen et al., 1987), cattle (MacAdam and Eberhart, 1972; Wagner and Oxenreider, 1972; Fulkerson et al., 1979, 1980; Thun et al., 1981; Lefcourt et al., 1993; Lyimo et al., 2000).

On the other hand, alterations of cortisol levels were found to be related to feeding times in goats (Eriksson and Teräväinen, 1989), and a circadian rhythm of plasma cortisol concentrations was observed in pregnant ewes fed once a day, but not in ewes fed throughout the day (Simonetta et al., 1991). Thus, ruminants likely have only a weak and low-amplitude intrinsic daily cortisol rhythm that is easily masked by external factors.

In goats, enhanced serum cortisol concentrations have frequently been measured in response to stress (e.g. Greenwood and Shutt, 1992; Kannan et al., 2000).

ANNUAL RHYTHM OF CORTISOL

Long-term lighting conditions play an important role in the seasonality displayed by many mammalian species in behaviour and physiology (Hastings et al., 1985). Besides the cyclic hormonal changes needed for the successful production of offspring (Karsch et al., 1984), another requirement of seasonal alterations in metabolism comes from variation of environmental conditions (temperature, humidity) and the availability of nutrients.

Seasonal changes in the activity and responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis have been reported in studies with rodents (Boswell et al., 1994), fish (trout, *Oncorhynchus mykiss,* McLeese et al., 1994), primates (squirrel monkey, *Saimiri sciureus,* Schiml et al., 1996) and humans (Walker et al., 1997). Seasonal changes in physiology and behaviour are associated with the breeding season, e.g. changes in the secretion of hormones involved in the reproductive (Lincoln and Kay, 1979; Suttie et al., 1992) and growth and metabolic (Suttie et al., 1989) axes. Increased aggressive behaviour and reduced voluntary food intake result in weight loss and an acute rise in cortisol and testosterone levels in male goats (Liptrap and Raeside, 1978; Howland et al. 1985). HPA axis activity can be modulated by changes in reproductive function (Bass et al., 1982; Verkerk and Macmillan, 1997), metabolic and growth demands (Yanovski et al., 1997) and social factors (Lyons et al., 1988).

Although some studies have demonstrated annual changes or photoperiodic modulations of glucocorticoid levels also in ruminants including sheep (Brinklow and Forbes, 1984), deer (Bubenik et al., 1977, 1983; Monfort et al., 1993; Feher et al., 1994; Ingram et al., 1999) and bulls (Leining et al., 1980), other investigations have failed to detect such alterations in white-tailed deer (Bubenik et al., 1975), axis deer (*Axis axis,* Bubenik and Brown, 1989) and rams (Kennaway et al., 1981; Lincoln et al., 1982). The variability of patterns has been suggested to depend on the age and sex of animals or on the social structure of the herd (Feher et al., 1994).

LEPTIN

Leptin (from the Greek word leptos, meaning thin) is a 16-kDa protein hormone (Zhang et al., 1994). It is mainly produced by adipose tissue, although smaller amounts of leptin have been shown to be produced by other cells and organs such as bone marrow and placenta (e.g. Hoggard et al., 1997; Laharrague et al., 1998; Margetic et al., 2002; Zhao et al., 2004).

Leptin is involved in regulating body weight, food intake, energy balance and reproduction (Ahima et al., 1997). It is one of the hormones that is tightly associated with lipid metabolism (Siegrist-Kaiser et al., 1997; Unger et al., 1999). The rates of leptin secretion and leptin plasma concentra-

tion are correlated with total fat mass (Hamilton et al., 1995; Maffei et al., 1995; Klein et al., 1996). The leptin receptor is expressed in various tissues such as the cerebral cortex, cerebellum, choroid plexus, lung, kidney, skeletal muscles, liver, pancreas, adipose tissue, adrenal medulla and especially in the ventromedial nucleus of the hypothalamus (VHM), known as the "satiety centre" (Tartaglia et al., 1995; Kieffer et al., 1996; Lee et al., 1996; Cao et al., 1997; Golden et al., 1997; Reidy and Weber, 2000). Circulating leptin levels inform the brain about the energy supply in order to regulate appetite and metabolism. It has been proposed that leptin`s primary role is to provide information to the hypothalamus, where the amount of energy stored in the adipose tissue is regulated. Daily administration of recombinant leptin decreases food intake, increases energy expenditures and promotes weight loss in mice (Campfield et al., 1995; Halaas et al., 1995, 1997; Pellymounter et al., 1995; Halaas and Friedman, 1997). Leptin acts by inhibiting the activity of neurons containing neuropeptide Y (NPY) and agouti-related peptide (AgRP), and by increasing the activity of neurons expressing α-melanocyte-stimulating hormone (α-MSH). AgRP functions as an endogenous antagonist of the anorectic effect of α-MSH at melacortin receptors (Lu et al., 1994; Fan et al., 1997; Ollmann et al., 1997; Takahashi and Cone, 2005; Arora and Anubhuti, 2006).

DAILY RHYTHM OF LEPTIN

Clear daily fluctuations in serum leptin levels have repeatedly been found in rats (Dallman et al., 1999; Kalsbeek et al., 2001), mice (Ahima et al., 1998; Ahrén et al., 2000) and humans (Sinha et al., 1996; Langendonk et el., 1998; Licinio et al., 1998), with a nocturnal increase and decrease at the end of the dark period and during light. A daily rhythm of plasma leptin levels has also been observed in fed Cosmina ewes (Bertolucci et al., 2005) and horses (Piccione et al., 2004; Buff et al., 2005), with a minimum during the light phase and a peak during the dark phase. In Syrian and Siberian hamsters, the results have been inconsistent (Drazen et al., 2000; Horton et al., 2000; Gündüz, 2002), and no circadian rhythm has been detected in sheep and Blackface ewes (Blache et al., 2000; Tokuda et al., 2000; Marie et al., 2001; Daniel et al., 2002).

ANNUAL RHYTHM OF LEPTIN

In rodents, leptin has been reported to be a powerful annual regulator of food intake and energy expenditure (e.g. Friedman and Halaas, 1998), and it has also been observed to have effects on reproduction (Ahima et al., 1996; Barash et al., 1996; Zhao et al., 2004; Moynihan et al., 2006).

Investigations of the photoperiodic regulation of leptin levels in ruminants have yielded contradictory results and led to different interpretations. Plasma leptin levels and leptin gene expression in perirenal adipose tissue were decreased in ovariectomized ewes exposed to short days, independently of the feeding regime (Bocquier et al., 1998). The authors concluded that leptin is modulated by day length independently of food intake, fatness and gonadal activity. Similarly, in ovariectomized cows, serum leptin levels were lower in winter than in summer, without changes in body weight (Garcia et al., 2002). In the Soay ram, the leptin levels were also lower under short than long days, but the difference was interpreted to depend on the photoperiod-induced changes in food intake and adiposity rather than on the direct effects of lighting on leptin secretion (Marie et al., 2001). In another study in Soay rams, no difference was found in serum leptin levels between long and short day-exposed animals, although the mean body weight was somewhat higher under long days (Clarke et al., 2003). In an earlier study by the same investigators, the serum leptin level tended to decrease over 16 weeks under short days and increase under long days, roughly paralleling the changes in body weight of the rams (Lincoln et al., 2001).

In seasonal-breeding mammals, there are fluctuations in leptin gene expression. In the Djungarian hamster, adipose tissue leptin gene expression was greatly reduced during winter or during exposure to a short photoperiod (Klingenspor et al., 1996). In ground squirrels, treatment with leptin just prior to hibernation resulted in a reduction in normal food intake and weight gain (Ormseth et al., 1996; Boyer et al., 1997). The decrease observed in leptin expression with decreasing photoperiod is probably an adaptive behaviour to decrease energy expenditure.

In the blue fox (*Alopex lagopus*), plasma leptin concentrations increased during autumn accumulation of fat and decreased during wintertime and the vernal weight loss period (Mustonen et al., 2005). In their study, leptin levels peaked 2-6 weeks before the maximum values were observed for voluntary food intake and body masses. The investigators suggest that leptin does not function as an acute indicator of body adiposity in seasonal carnivores but rather as a long-term signal of nutritional status.

LIPID METABOLISM

Fatty acids are an important source of energy for many organisms. Triglycerides (also known as triacylglycerols or triacylglycerides) are glycerides in which the glycerol is esterified with three fatty acids. The breakdown of fat stored in fat cells is known as lipolysis (hydrolysis by lipases). Triglycerides are broken down into glycerol and free (or non-esterified) fatty acids (FFA or NEFA) by lipases with the help of bile salts. The following hormones

induce lipolysis: adrenaline, noradrenaline, glucagon and adrenocorticotropic hormone (e.g. Voet and Voet, 2002).

Triglycerides yield more than twice as much energy for the same mass as carbohydrates or proteins. All cell membranes are made of phospholipids, each of which contains two fatty acids, proteins and cholesterol. Fatty acids are also commonly used for protein modification, and all steroid hormones are ultimately derived from fatty acids. The metabolism of fatty acids, therefore, consists of catabolic processes which generate energy and primary metabolites from fatty acids, and anabolic processes which create biologically important molecules from fatty acids and other dietary carbon sources (e.g. Heideman, 2002).

Lipid metabolism in ruminants

A ruminant is any hooved animal that digests its food in two stages. After initial eating, the animal regurgitates semi-digested food known as cud, which is then rechewed. The process is called ruminating. Ruminants include cattle, goats, sheep, giraffes, bison, buffaloes, deer, wildebeest, and antelopes (e.g. Eckert et al., 1988).

Ruminants′ stomachs consist of four chambers: the rumen, reticulum, omasum and abomasum. In the first two chambers, the rumen and the reticulum, the food is mixed with saliva, and the cud (or bolus) is formed. The cud is then regurgitated, chewed slowly to further break down the food particles and mix it with saliva. Fibre, especially cellulose, is broken down into glucose in these chambers by symbiotic bacteria and protozoa. The broken-down fibre particles, which are now in the liquid part of the contents, then pass through the rumen into the next stomach chamber, the omasum, where water is removed. After this, the digesting food is moved to the last chamber, the abomasum. The food in the abomasum is digested much like it would be in the human stomach. It is finally sent to the small intestine, where the absorption of nutrients occurs (e.g. Herdt, 2002).

Almost all the glucose produced by the breaking down of cellulose is used by the symbiotic bacteria. Ruminants differ from single-stomached animals in that dietary carbohydrates are degraded in rumen to hexoses and pentoses, which are then fermented by micro-organisms to produce short-chain volatile fatty acids (acetate, propionate, butyrate, reviewed e.g. by Hocquette and Bauchart, 1999). These fatty acids are absorbed through the rumen wall by simple diffusion. Acetate, not metabolized by the liver to any great extent, is distributed to other tissues to be used as an energy source and substrate for lipogenesis in the adipose tissue. Although 80-90% of propionate and butyrate is removed in a single pass through the liver to be further metabolized for energy requirements, a concentrate meal is associated with a rise of the level of the volatile fatty acids in peripheral plasma within 2-4 h (Bassett, 1974; Sutton et al., 1988; Blum et al., 2000). Triacylglycerols are also hydrolysed by rumen lipases, but most of the ab-

sorption of medium- and long-chain fatty acids occurs in the jejunum after interaction with bile and pancreatic lipase. Plasma concentration of FFA in ruminants increases before the morning meal and decreases rapidly after the meal (Blum et al., 1985; 2000; Marie et al., 2001).

Rumination occurs when the animal is not actively eating, usually during times of rest, but not during deep sleep. The time spent ruminating depends on the type of diet and appears to range from almost none for high-grain diets to a maximum of about 10 h per day for high-forage diets. The feed intake level also influences the amount of rumination time, with high intakes stimulating greater rumination (e.g. Herdt, 2002).

DAILY RHYTHMS OF FREE FATTY ACIDS (FFA) AND GLYCEROL

Daily rhythmicity of lipid metabolism has been described in some animal species when the animals are fed *ad libitum*. Regular daily fluctuations of plasma concentrations of FFA have been observed in rats (Escobar et al., 1998; Dallman et al., 1999; Tsutsumi et al., 2002), sheep (Marie et al., 2001) and cattle (Blum et al., 1985, 2000; Zanzinger et al., 1994). In contrast to the above studies, no significant daily rhythmicity of FFA levels was found in dairy cows with free access to food (Bassett, 1974; Sutton et al., 1988; Bitman et al., 1990). The daily changes of FFA concentrations occurring during voluntary feeding behaviour have been explained to be due to the intervals between eating because lipolysis during fasting increases the plasma FFA levels. Rapid changes in the lipolysis/lipogenesis balance have been demonstrated in experiments in which feeding was restricted in rats (Escobar et al., 1998; Dallman et al., 1999), sheep (Bassett, 1974; Marie et al., 2001) and cattle (Blum et al., 1985).

Besides meal times, other factors can contribute to maintenance of the daily rhythm of lipid metabolism. During extended fasting in rats, the 24-h rhythmicity of FFA concentrations disappeared at first, but after 48 h of fasting the rhythm reappeared (Escobar et al., 1998). In ruminants receiving food portions twice daily, the rise and fall of plasma FFA levels were associated with the morning meal only, or the peak was more pronounced in the morning than in the afternoon (Bassett, 1974; Blum et al., 1985, 2000; Zanzinger, 1994; Marie et al., 2001). The FFA response to adrenaline injections in cows was also more pronounced in the morning than in the evening (Fröhli and Blum, 1988).

SEASONAL VARIATION OF LIPID METABOLISM

In addition to daily variations, metabolism undergoes pronounced seasonal fluctuations in many species of mammals (for review, see Lincoln and Richardson, 1998; Clarke, 2001; Bartness et al., 2002; Rhind et al., 2002). These fluctuations are mainly triggered by the photoperiod, but the exact mechanisms underlying this phenomenon are not known, and considerable species differences exist in the regulatory systems (Clarke, 2001).

In ruminants in semi-natural conditions, plasma concentrations of FFA and glycerol of the reindeer (*Rangifer tarandus tarandus*) were found to be relatively low in summer, suggesting high lipogenic activity (Larsen et al., 1985), whereas in the Alaskan reindeer, FFA concentrations fluctuated throughout the year without any clear trend (Bubenik et al., 1998). Serum total lipid and triglyceride concentrations in Finnish reindeer were twofold in fall compared with the concentrations in winter and spring (Nieminen et al., 1984). In artificial lighting conditions, sheep with freely available food gained about 10 kg during 16 weeks in a long-day condition after an equal period under short days, and their plasma FFA levels were significantly lower at the end of long-day exposure than short-day exposure (Marie et al., 2001). FFA concentrations were also significantly lower in long- than in short-day-exposed underfed ewes, suggesting enhanced fat mobilization when underfeeding occurred during short days (Bocquier et al., 1998). Variations of enzyme activity in adipose and muscle tissues of sheep living under short or long photoperiods demonstrated that these domesticated animals have preserved the ability to anticipate seasonal changes in food resources even when the food intake is kept constant (Faulconnier et al., 2001).

AIMS OF THE STUDY

This thesis examined the effect of daily and annual lighting conditions on the blood levels of the hormones melatonin, cortisol and leptin and the lipid metabolism of free fatty acids (FFAs) and glycerol in female Finnish landrace goats. Blood samples were collected in six seasons of the year during artificial lighting conditions approximately simulating the annual changes of day length at 60°N. Ambient temperature and feeding regime were kept constant. The rhythms were characterized also in constant darkness after each lighting regime in order to differentiate between a direct effect of light and other regulatory mechanisms.

More specific aims were as follows:

1. To determine whether significant seasonal differences exist in endogenous melatonin profiles, in addition to the expected seasonal differences in the profiles adjusted directly by light.

2. To investigate whether the serum cortisol levels of goats display daily and/or seasonal rhythmicity, and whether the concentration profiles differ between normal light/dark and constant dark conditions.

3. To determine how the lipid metabolism of goats is related to the daily and seasonal variations in lighting conditions and the feeding schedule, and whether possible changes in lipid metabolism are related to leptin levels.

4. To evaluate whether the daily FFA rhythm is influenced by endogenous melatonin rhythm.

MATERIALS AND METHODS

Details for all experiments are given in the original publications (I–IV). The main principles are described below.

Ethical considerations

All experiments were carried out in accordance with the laws of Finland and the European Convention for the protection of experimental animals (No. 1360/1990) and with the approval of the Local Ethics Committee for animal experimentation.

Animals

The domestic goat (*Capra aegagrus hircus*) was first documented in the highlands of western Iran 10000 calibrated calender years ago (Zeder and Hesse, 2000).

The goat is a member of the Ruminantia and is closely related to the sheep. They eat plants, including pastures and many weeds, shrubs and trees. They digest food in two steps, chewing and swallowing normally, then regurgitating the semi-digested cud in order to re-chew it. This process is called rumination. Digestion is a process of breaking down this plant material in the stomach and intestine into components that can be absorbed and used by the goat. The stomach of the goat is very large and consists of four parts: the rumen, the reticulum, the omasum and the abomasum (e.g. Herdt, 2002).

Goats mature sexually at 5-8 months of age. The photoperiod controls the occurrence of reproductive cycles in goats. They have an annual period in which they have continuous (cyclical) ovarian activity and another period of no ovarian activity. The ovarian activity is positively affected by decreasing photoperiod. The main translator of the photoperiod is the pineal gland, which produces melatonin in response to darkness (e.g. Cunningham, 2002).

Finnish landrace goats (*Capra hircus*) are located primarily in western Finland. They are kept mainly for milk production. Both polled and horned individuals. The usual colour is white, but grey and pied variants also exist are found. The length of the hair varies.

The experimental animals comprised seven adult female Finnish land-

race goats. The mean age of the animals at the beginning of the experiments, which lasted two years, was 9 years, range 5 – 13 years. The mean weight was 50 kg, range 46 – 54 kg. All goats were clinically healthy, non-pregnant and non-lactating. The goats were kept in pens and provided with hay, straw and water *ad libitum*. Hay was replenished twice daily at 0630 h and 1500 h. In addition, the animals were given two concentrate meals daily at 0700 – 0730 h and 1200 – 1230 h. Each meal consisted of a mixture of oats, energy content 11.5 MJ/kg (60 g/portion) and grained forage, energy content 12.3 MJ/kg (15 g/portion, Lammas-Mella, Lännen Tehtaat Oyj, Iso-Vimma, Finland). The noon meal was completed with a piece of carrot or an other fresh delicacy.

The goats were housed in three identical experimental rooms, with 3, 2 and 2 animals in each. Before the experiments, the goats had been kept from birth under natural indoor lighting in winter and outdoors in summer.

General experimental procedures

Lighting conditions

During the experiments, the animals were kept in artificial lighting that approximately simulated the annual changes of natural photoperiod in Helsinki, Finland (60°N) (Figure 2). This lighting condition is henceforth referred to as a light/dark (LD) condition. The light and dark periods were shortened and prolonged at 2- to 6-week intervals, symmetrically maintaining the midpoints at noon and midnight, respectively. The temperature in the pens was 18 – 23°C throughout the year. For about 2 months in summer (June, July), the animals grazed outdoors under natural lighting and temperature conditions (local mean temperature about 15 °C and 17°C in June and July, respectively).

The lighting conditions were as follows: early fall, LD 14:10 (lights on from 0500 to 1900 h); late fall, LD 10:14 (0700 to 1700 h); winter, LD 6:18 (0900 to 1500 h); early spring, LD 10:14 (0700 to 1700); late spring, LD 14:10 (0500 to 1900 h); summer, LD 18:6 (0300 to 2100 h). During the light period, the illuminance was 100 to 150 lux at the level of the goats´ heads (warm white fluorescent tubes, Osram, Augsburg, Germany). During the dark period and continuous darkness, there was no light, except during the sampling days, when dim red incandescent lamps (<1 lux) were kept on. There was a half-year interval between the first- and second-year sample collection periods; during this period, the animals were kept under 12:12 h LD conditions.

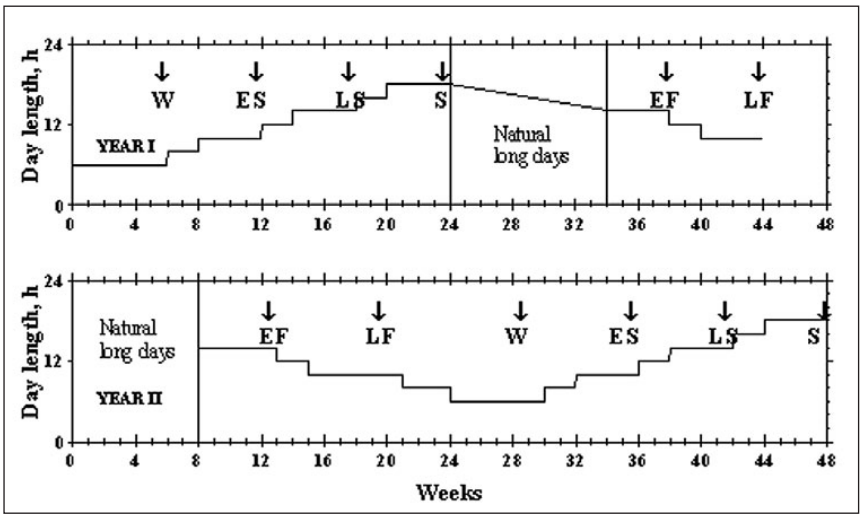

Figure 2. Lighting conditions during the experiment. Sampling weeks during the two years are indicated by arrows. W = winter, ES = early spring, LS = late spring, S = summer, EF = early fall, LF = late fall. The first day of week 0 was 17 December for the first year and 25 June for the second year. Samples were collected during the first year for melatonin (I) and cortisol (II), during the second year for free fatty acids (III, IV), glycerol (III) and melatonin (IV) and during the first and second years for leptin (III) measurements. (Reprinted from Comparative Biochemistry and Physiology, Part A, Vol. 138, Alila-Johanssson et al., Daily and annual variations of free fatty acid, glycerol and leptin plasma concentrations in goats (Capra hircus) under different photoperiods, pp. 119-131, 2004, with permission from Elsevier.)

Sampling

Blood samples were collected during two consecutive years (6 times/year) after the respective lighting regime had been maintained for at least 24 days. Blood samples were taken via a plastic catheter inserted into the jugular vein under local anaesthesia 2-3 h before the first sampling. The catheter was flushed with sodium citrate solution after sampling. Blood samples were collected at 2-h intervals for 2 days, first in LD conditions and then in constant darkness, either in the first day (year I) or after 3 days (year II). Blood samples were taken into glass and EDTA tubes. Samples were centrifuged and serum was stored at -20°C until assayed for melatonin, cortisol, leptin and progesterone concentrations. Plasma was stored at –70°C until assayed for free fatty acids and glycerol.

All goats were sampled within 5-10 min and the same order of sampling was maintained throughout the study. All samples of a goat were measured in the same assay. Lighting conditions and blood sampling procedures were similar for both experimental years. The summer samples were collected before the animals were put out to grass and the early fall samples after the grazing period.

Melatonin

Melatonin was extracted from 1.0 ml of serum with chloroform and measured in duplicate by radioimmunoassay (Vakkuri et al., 1984; Laakso et al., 1988). The radioimmunoassay (RIA) uses [^{125}I]-melatonin as a tracer for determination of melatonin in serum. Melatonin antiserum was produced in the rabbit by immunization with bovine thyroglobulin conjugate of *N*-acetyl-5-methoxytryptophan. The cross-reactivity of antiserum was 15% determined with *N*-acetyl-tryptophan, 9% with 5-methoxytryptamine, 0% with 6-hydroxymelatonin and 0% with 5-methoxytryptophan (50 pg/tube each). The final tube dilution of 1:2400 resulted in 30-40% binding in the samples without cold melatonin. All samples of each season were measured in the same assay. The non-specific binding of the tracer was 5-6%. The least detectable concentration, defined as apparent concentration at 2 standard deviations from the counts at maximum binding (n=6 in each assay), was smaller than the lowest standard (1.95 ng/l). The intra-assay variability calculated from the duplicate measurements was 5-13%. The interassay variability in the 12 assays of this study was 8-15%, depending on the concentration.

Cortisol

Serum concentrations of cortisol were measured by RIA with Coat-A-Count RIA kits obtained from Diagnostic Products Corporation (Los Angeles, CA). Cortisol was measured in 25-µl duplicates. Additional lower calibration points were prepared using zero calibrator as diluent. The non-specific binding of the tracer was 1-2%. The least detectable concentration was smaller than the lowest standard (8.63 nmol/l). Intra-assay variability calculated from the duplicate measurements was 9-13% at the concentration range of the samples. The interassay variability in the 8 assays of this study was 15% and 10% at concentrations of 9 and 76 nmol/l, respectively.

Leptin

Serum concentrations of leptin were determined using the Linco Multispecies Ria Kit (Cat. # XL-85K, Linco Research, Inc., St. Charles, MO). The ability of the assay kit to quantitatively recover leptin in goat serum was evaluated by adding 50 ng/ml of purified recombinant human leptin to a goat serum pool. The serum pool samples were diluted 1:2, 1:4, 1:8, 1:16, and 1:32. Recovery ranged from 80% to 128% of expected values, and the line paralleled the standard curve. The calibration range of the assay was 1.0 – 50.0 ng/ml. Intra-assay variability calculated from the duplicate measurements was 4-7% at the concentration range of the samples. Interassay variability in the eight assays of this study was 7-10% at concentrations of 4.0 and 28.0 ng/ml, respectively.

Progesterone

Serum progesterone was measured by RIA(Coat-A-Count RIA kit, Diagnostic Products Corporation, Los Angeles, CA) in 100-µl duplicates. The non-specific binding of the tracer was <1%, intra-assay variability 5-8% and interassay variability in the six assays of this study 6% and 9% at concentrations of 1.5 and 12 ng/ml, respectively. In experimental year I, progesterone was measured in all 13 samples of each sampling day. In year II it was measured in the first and last samples of each sampling.

FFA and glycerol

Plasma concentrations of FFAs and glycerol were determined in the plasma samples of year II by using a fully automated KONE Specific Analyser. FFAs were measured by commercial photometric enzymatic assays (Wako, Neuss, Germany). Intra-assay variability, calculated from eight measurements of a control sample of 1.0 mmol/l, was 1.2%. Interassay variability in the nine assays of this study was 4.4% at a concentration of 0.1 mmol/l.

Plasma glycerol concentrations were measured using commercial photometric enzymatic assays (Triglyceride, GPO-TRINDER, Methode-Nr. 337 Sigma Diagnostics). Intra-assay variability, calculated from eight measurements of a control sample of 275 µmol/l, was 0.13%. The interassay variability in the nine assays of this study was 1.2-6.4%, depending on the concentration.

DEFINITIONS AND CALCULATIONS

Characteristics of daily melatonin rhythm

Melatonin onset and offset times were determined for each individual daily pattern graphically without smoothing. Due to a very large interindividual variation of the melatonin levels, the determination was not possible by applying any fixed critical concentration, which is often used in, for instance, human studies (Lewy et al., 1999). Instead, the critical concentration for each individual goat was defined as 25% of the average concentration during the dark periods in LD conditions. The range of the thresholds was 5-16 pg/ml. The melatonin onset and offset times, both in LD and DD conditions, were defined as the time points at which the threshold concentration was reached at the rising and declining limbs of the daily pattern. The figure 25% was chosen because low percentages (e.g. 10%) were unreliable due to the lower accuracy of the assay at low concentrations, and higher percentages (e.g. 50%) produced an obvious bias in several patterns due to the episodic nocturnal secretion profiles in individual goats.

Duration of high melatonin levels (peak duration) was calculated as an interval between melatonin onset and offset times. In addition, intervals between melatonin onset times and (habitual) lights-off times and between melatonin offset and lights-on times were calculated.

Characteristics of daily FFA and glycerol rhythms

FFA trough, FFA peak and glycerol trough times were visually determined from the individual concentration profiles without smoothing. The FFA trough was the time of the lowest value in the profile, the FFA peak was the time of the highest value between midnight and noon and the glycerol trough was the time of the lowest value between midnight and noon.

The FFA peak level was the highest concentration in the morning, and the FFA trough level was the mean of the three lowest concentrations of the pattern.

The FFA half-rise time was the time point at which 50% of the amplitude was reached during the rising phase in the morning. The amplitude was the difference between the peak level and the trough level.

Daily mean levels and area-under-curve (AUC) values

The individual peak level of melatonin was defined as the mean of the three highest concentrations of the profile, with the exception that the summer peak was defined as the highest concentration of the profile.

The daily mean FFA, glycerol and leptin concentrations were calculated as means of individual mean levels.

Areas under the individual 24-h melatonin and cortisol curves (AUC values) were calculated for comparisons of the total amount of melatonin and cortisol in the circulation over the day.

STATISTICS

Repeated measures two-way analysis of variances (ANOVAs) followed by parametric post-tests (Tukey-Kramer or Bonferroni's tests for selected pairs) were used to determine possible differences in the hormone and lipid profiles among the seasons and between LD and DD conditions. Repeated measures one-way ANOVAs followed by Tukey's test were applied to evaluate the effect of time in single curves. Logarithmic transformation was used to reduce the inhomogeneity of variances. Linear regression models and Pearson's or Spearman's rank order correlation coefficients were applied to test the relationship between two groups of samples.

RESULTS

The main results of the thesis are presented below. Details of the results for all experiments are given in the original publications (I–IV).

DAILY AND ANNUAL PATTERNS OF SERUM MELATONIN (I and IV)

Melatonin profiles

During both first (Figure 3) and second (Figure 11) year in LD conditions, the average serum melatonin patterns of the goats were related to the length of the dark period. During all seasons, a rapid rise of serum melatonin concentrations occurred within 1 h after lights-off. The concentrations remained high until the lights were turned on. The only exception was the 18-h scotoperiod in winter. Then, all animals had low melatonin levels already 1–2 h before lights-on at 0800 h.

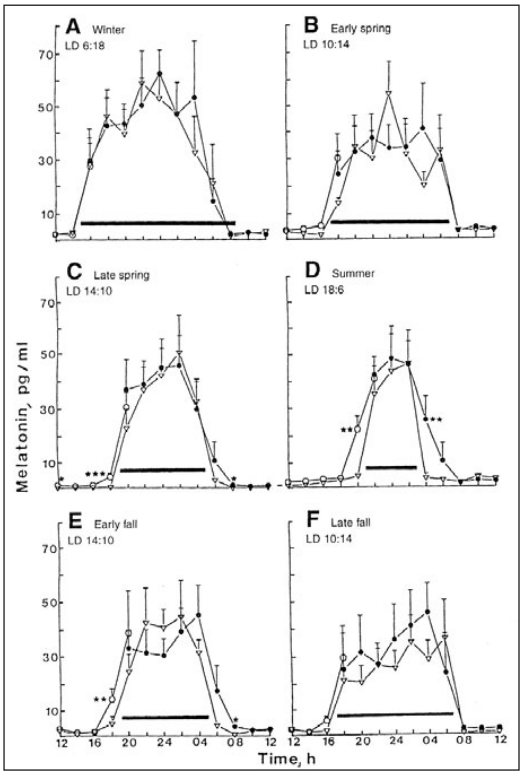

Figure 3 (A-F). Daily serum melatonin profiles in seven goats under various photoperiods during the course of a year. Seasons and hours of light and dark (LD) are given in the figure. The animals were kept in artificial lighting simulating annual natural photoperiods. Blood samples were collected at 2-h intervals after the respective lighting regime had been maintained at least for 4 weeks. Means with SEMs are shown. Open triangles = sampling in light/dark conditions from 12.00 to 12.00 h, filled circles = sampling in constant darkness beginning one hour after lights-off, open circles = continuation of the sampling in constant darkness. The black line above the abscissa denotes the habitual dark period. * different from the corresponding value in LD conditions, $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Bonferroni test for selected pairs). (Reprinted from Journal of Biological Rhythms, Vol. 16 (3), Alila-Johanssson et al., Seasonal variation in endogenous serum melatonin profiles in goats: a difference between spring and fall, pp. 254-263, 2001, with permission from Sage Publications.)

In DD conditions during year I (1 day in constant darkness), the melatonin concentrations at 1600 h were clearly elevated in winter, whereas in all other seasons the daytime values were low. In the DD conditions, the summer pattern differed from the respective patterns in winter, early spring and late fall, but not from the patterns in late spring and early fall.

In DD conditions during year II (3 days in constant darkness), the melatonin patterns were very similar to those observed in the habitual LD cycles, but they tended to advance. The advance of the average profile was significant only in summer. The comparisons of the melatonin profiles in DD among the seasons suggested that some of the differences in timing present in LD had disappeared in DD. The DD profile in winter was exceptional due to the very early rise of melatonin, and it was different from all other profiles.

During year I, the melatonin profiles in LD and DD conditions did not differ significantly from each other in winter, early spring or late fall (LD 6:18 or 10:14). In late spring, summer and early fall (LD 14:10 or 18:6), the patterns in DD were significantly "broader" than in LD. The rise of melatonin started earlier and the decline occurred later.

In year II the comparisons of the melatonin profiles in DD among the seasons suggested that some of the differences in timing found in LD had disappeared in DD; the patterns under the long habitual photoperiods (late spring, summer, early fall) were not significantly different from each other, nor were the patterns in early and late fall different. The profile in winter remained exceptional due to the very early rise of melatonin, and it was different from all other profiles (interaction season x Time $p<0.005$ or less in all comparisons).

Melatonin rhythm characteristics: light-dark (LD) versus continuous darkness (DD)

In LD conditions, melatonin onset times coincided with the lights-off time in both year I and II. In DD conditions (year I), the onset time remained unchanged only in winter; in all other seasons, the onset advanced significantly after 1 day in darkness. When the habitual scotoperiod was 18 or 14 h (winter, early spring, late fall), the clock times of melatonin onset in DD conditions were not significantly different from each other. The onset time in summer did not differ from that in late spring, but was different from all other onset times, including that in early fall.

In LD conditions, melatonin offset times occurred mostly around the lighting transition in both year I and II. The offset time in winter was an exception; on average, the defined critical level of melatonin was reached almost 4 h before the lights-on time.

In DD conditions (year I), the offset times were 5.3 – 6.7 h after midnight with large interindividual variances, and they were not significantly affected by the seasons. The only significant shift of melatonin offset in the

morning before the lights were switched on was the delay occurring in summer.

In equal habitual LD conditions in late spring and early fall (LD 14/10), the endogenous melatonin rhythms were not very similar; the pattern in late spring resembled that in summer and the pattern in early fall that in winter.

In DD conditions (year II), both the melatonin onset and offset tended to advance. The mean advance of melatonin offset was significant under the longest scotoperiods in late fall, winter and early spring.

In equal habitual LD conditions in late spring and early fall (LD 14/10), the difference in endogenous melatonin rhythms had disappeared.

Melatonin levels and rhythm characteristics: seasonal variation

In LD conditions (year I), the period of high melatonin concentrations (>25% of the dark-time mean level) corresponded to the length of the dark period in spring and fall, when the darkness lasted 14 or 10 h (Figure 4, Table 1). In summer, it was clearly longer than the 6-h scotoperiod, reflecting the very steep rise after lights-off and the abrupt decline after lights-on. In winter, the peak duration was shorter than the 18-h scotoperiod and did not differ from the durations under the 10:14 LD conditions in early spring and late fall.

In DD conditions, the peak durations did not differ from the corresponding values in LD conditions, when the habitual scotoperiod was 14 or 18 h, nor were the durations different in late spring, although the habitual scotoperiod was shortened to 10 h. In early fall, however, the mean peak duration was significantly longer in DD than LD conditions, irrespective of the same 10-h habitual scotoperiod. The most marked prolongation of the peak duration in DD conditions was found in summer (6-h scotoperiod).

In LD conditions (year I), the melatonin onset time coincided with the lights-off time in all seasons, except in summer, when the defined onset level was reached before the lights-off time. In DD conditions, the melatonin onset time tended to advance, occurring significantly before the habitual lights-off time in all seasons except winter.

In early spring and late fall, when the scotoperiod was 14 h, the melatonin offset time coincided with the lights-on time in both LD and DD conditions. In winter, under the 18-h scotoperiod, the duration of the melatonin peak was also about 14 h, resulting in the occurrence of melatonin offset times significantly before the lights-on time.

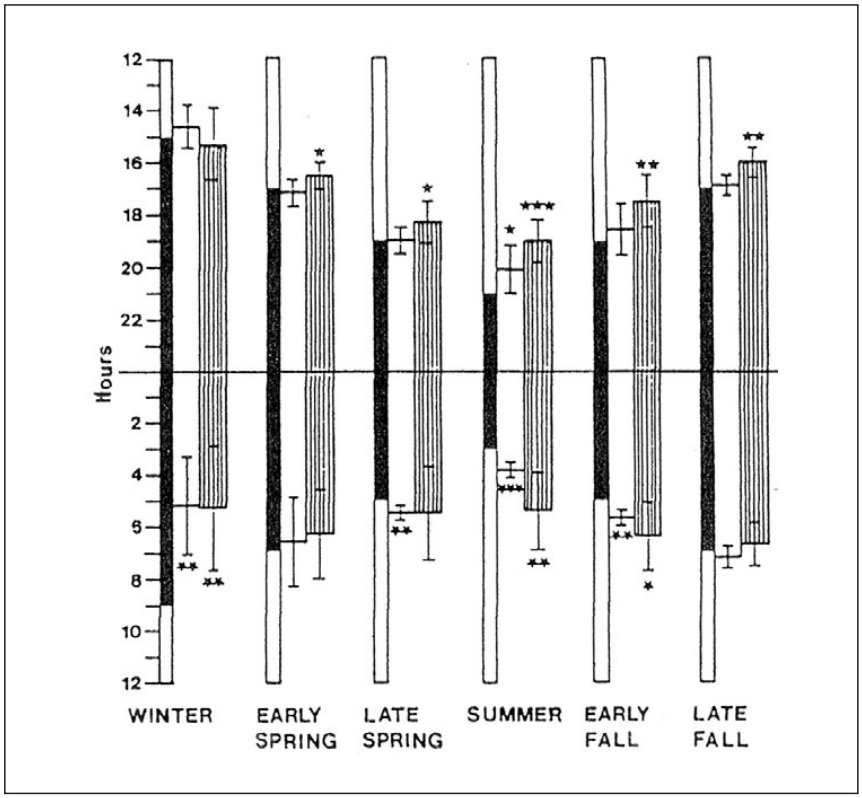

Figure 4. Relationships of melatonin onset and offset times (means with SD:s) to the lights-off and lights-on times in seven goats under various photoperiods during the course of a year. The periods of high melatonin levels (Table 1) in relation to the respective scotoperiod are visualized: black columns = scotoperiod, white columns = period of high melatonin levels in light-dark (LD) conditions, hatched columns = in continuous darkness (DD). Melatonin onset and offset times were determined for each animal according to an individual threshold melatonin level (see Materials and Methods). * different from the respective illumination transition time, $p<0.05$, ** $p<0.01$, *** $p<0.001$ (one-sample t-test). (Reprinted from Journal of Biological Rhythms, Vol. 16 (3), Alila-Johanssson et al., Seasonal variation in endogenous serum melatonin profiles in goats: a difference between spring and fall, pp. 254-263, 2001, with permission from Sage Publications.)

Table 1. Duration of high melatonin serum levels (hours, mean±SEM) in seven goats under various photoperiods during the course of a year. The melatonin profiles were determined in light/dark conditions (LD) and on the following day in continuous darkness (DD). The durations were calculated as intervals between the melatonin onset and offset times. Duration differences between LD and DD conditions are also given. (Reprinted from Journal of Biological Rhythms, Vol. 16 (3), Alila-Johansson et al., Seasonal variation in endogenous serum melatonin profiles in goats: a difference between spring and fall, pp. 254-263, 2001, with permission from Sage Publications.)

		Duration of high melatonin level (h)		Difference
Season	**L:D (h)**	**LD**	**DD**	**DD–LD**
Winter	6:18	14.6 ± 0.6	14.0 ± 0.7	–0.6 ± 0.5
Early spring	10:14	13.5 ± 0.6	13.8. ± 0.7	0.3 ± 0.3
Late spring	14:10	10.5 ± 0.2	11.2 ± 0.6	0.7 ± 0.7
Summer	18:6	7.7 ± 0.4	10.5 ± 0.5	2.8 ± 0.7**
Early fall	14:10	11.1 ± 0.4	13.0 ± 0.5	1.9 ± 0.4**
Late fall	10:14	14.3± 0.3	14.7 ± 0.3	0.4 ± 0.3

NOTE: Two-way ANOVA: LD versus DD $p<0.05$, season $p<0.001$, interaction $p<0.001$. One-way ANOVA for LD values $p<0.0001$, for DD values $p< 0.0001$; for more detailed comparisons, see the text. ** different from zero, $p < 0.01$ (one-sample *t* test).

Under the shorter scotoperiods (10 h in late spring and early fall, 6 h in summer), the melatonin offset occurred only after the lights-on times, probably reflecting a steep light-induced decline in the concentrations. In late spring, the offset time in DD conditions was equal to that in LD conditions, but due to the large interindividual variation, the melatonin offset did not differ significantly from the habitual transition time of lighting. In summer and early fall, the melatonin offset times were delayed in DD conditions and, irrespective of the large interindividual variations, the means were different from the habitual lights-on times.

DAILY AND ANNUAL PATTERNS OF SERUM CORTISOL (II)

Cortisol profiles in LD and DD

There was no significant daily rhythm in the serum cortisol levels of the goats at any time of the year (Figure 5). Neither did the profiles in LD and DD conditions differ significantly from each other. In winter, the high mean values after midnight in LD conditions were mainly caused by the values of one goat. At no time of the year did the cortisol levels in LD and DD differ from each other at the time points immediately after the turning on or off of lights.

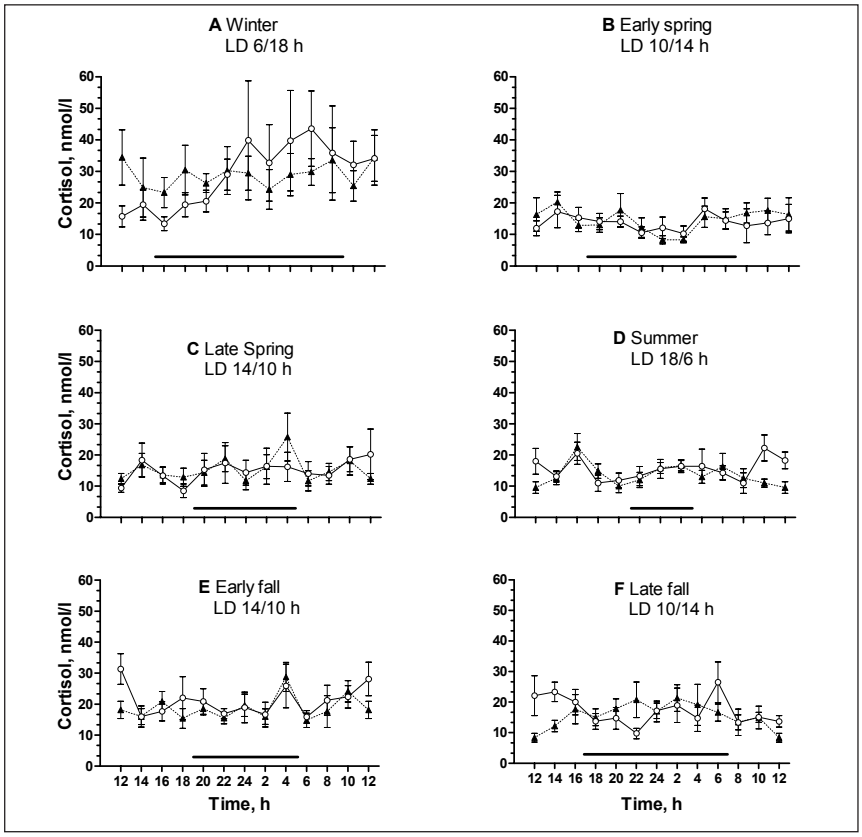

Figure 5 (A-F). Daily cortisol profiles in seven goats under various photoperiods during the course of a year. Times of year and hours of light and dark (LD) are given in the figure. The animals were kept in artificial lighting simulating annual natural photoperiods. Blood samples were collected at 2-h intervals after the respective lighting regime had been maintained for at least 4 weeks. Means with SEMs are shown. Open circles = sampling in light:dark conditions from 1200 to 1200 h, filled triangles = sampling in constant darkness beginning 1 h after lights-off. The black line above the abscissa denotes the habitual dark period. One-way ANOVA for each curve: NS. Two-way ANOVA for each pair of curves in any season (LD, DD): lighting NS, time NS, interaction NS. (Reprinted from Chronobiology International, Vol. 20, Alila-Johanssson et al., Serum cortisol levels in goats exhibit seasonal but not daily rhythmicity, pp. 65-79, 2003, with permission from the Marcel Dekker, Inc.)

Cortisol concentrations in different seasons

Significant seasonal differences existed among the overall cortisol levels during the year. In winter, the concentrations were higher than at any other time of the year. The cortisol concentration was at its lowest from early spring to summer. In early fall, the concentrations were significantly elevated in both LD and DD conditions compared with early spring, late spring and summer. Also in late fall in LD conditions, the levels were higher than in early spring. The large variances in winter and late fall were caused by high variations in the individual overall levels among the animals. The daily fluctuations in single animals were similar throughout the year.

Leptin profiles in LD and DD

Serum leptin concentrations displayed no daily rhythm in any of the seasons studied. Neither were there any significant differences in the profiles in LD and DD conditions (Figure 6).

The overall leptin levels in LD conditions were significantly lower in early and late fall than in early spring. In DD conditions, the overall level in early fall was lower than in winter, early spring and summer, and the level in late fall was lower than in summer.

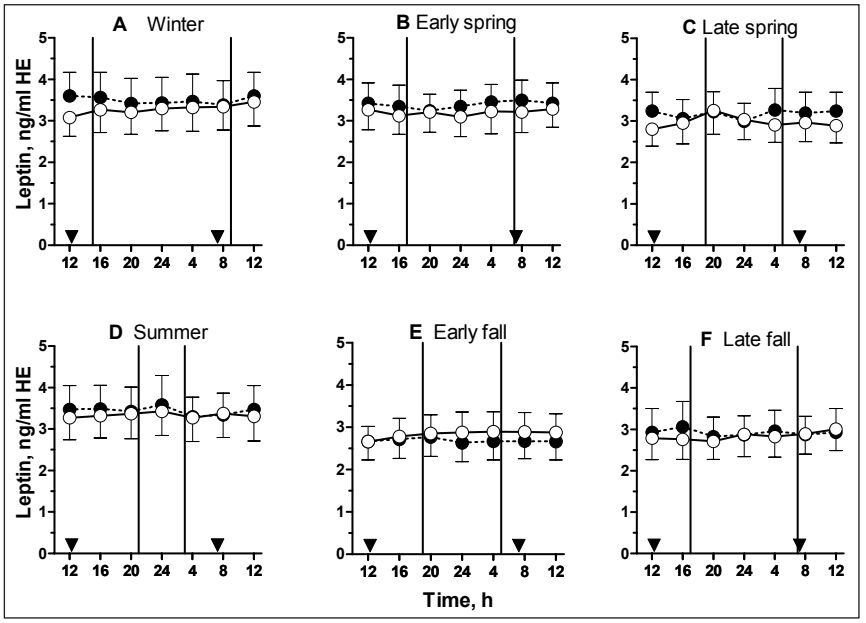

Figure 6 (A-F). Twenty-four-hour patterns of serum leptin concentrations in seven goats under various photoperiods during year I (means with SEMs). The animals were kept in artificial lighting simulating annual natural photoperiods. The habitual dark period is shown as an interval between the vertical lines in each figure. Leptin concentrations were measured in samples collected at 4-h intervals after the respective lighting regime had been maintained for at least 4 weeks. Open circles = sampling in light-dark (LD) conditions from 1200 to 1200 h, filled circles = sampling on the first day of continuous darkness (DD) beginning 1 h after the habitual lights-off. HE = human equivalents. Triangles on the abscissa denote concentrate feeding times. One-way ANOVA for each curve: NS. Two-way ANOVA for each pair of curves (LD, DD) at all times of year: lighting NS, time NS, interaction NS. (Reprinted from Comparative Biochemistry and Physiology, Part A, Vol. 138, Alila-Johanssson et al., Daily and annual variations of free fatty acid, glycerol and leptin plasma concentrations in goats (*Capra hircus*) under different photoperiods, pp. 119-131, 2004, with permission from Elsevier.)

Leptin concentrations in different seasons

During both experimental years, both in LD conditions and in DD conditions, the daily mean levels of leptin tended to be low in early fall and high in winter. Seasonal differences were rather small, and only during the first year in DD was the daily mean level in early fall significantly lower than in winter. No differences were present in the overall daily mean levels between LD and DD conditions, but the levels were higher in year I than in year II.

In year II, at the time of the sampling, the mean mass of the animals in early fall was significantly lower than at any other time of the year. Thus, serum leptin levels tended to be low when the mass of the goats was at its lowest. There was, however, no significant correlation between the daily mean leptin level and the mean mass of the animals measured at different times. It is noticeable that only one of the data points (early fall) deviated significantly from the other points.

The interindividual variation of the annual mean leptin levels was large (range in year II 1.1–4.1 ng/ml). The annual mean masses of the individual animals also varied considerably (range 42.9 – 61.2 kg). A positive correlation was apparent between the mean leptin level and the mean mass of individual animals.

DAILY AND ANNUAL PATTERNS OF PLASMA FFA AND GLYCEROL (III)

FFA and glycerol concentration profiles in LD and DD

The daily variation in plasma free fatty acid (FFA) concentrations of the goats was significant in all seasons. In both LD and DD conditions a constant rise of concentration occurred early in the morning after low levels at night. The amplitude of the morning peak was somewhat higher in winter and spring than in summer and fall (40–60% vs. 10–30% above the daily mean level). After 3 days in at the beginning of the sampling in the afternoon, some goats had exceptionally high FFA levels in early and late fall (Figure 7). The overall levels did not, however, differ between LD and DD conditions at any time of the year.

In LD conditions, overall levels of FFA were low in summer and fall and high in winter and spring. The levels were significantly lower in summer than in early spring and late spring, and the levels were lower in late fall than in winter, early spring and late spring. In DD conditions, the levels were lower in summer than in winter, early spring and late spring.

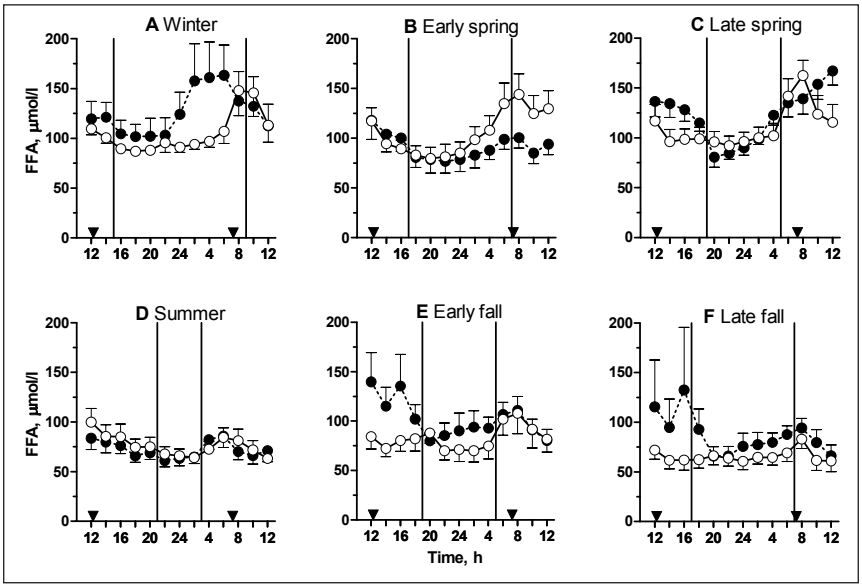

Figure 7 (A-F). Twenty-four-hour patterns of plasma free fatty acid (FFA) concentrations in seven goats under various photoperiods during year II (means with SEMs). The animals were kept in artificial lighting simulating annual natural photoperiods. The habitual dark period is shown as an interval between the vertical lines in each figure. Blood samples were collected at 2-h intervals from 1200 to 1200 h after the respective lighting regime had been maintained for at least 24 days. Open circles = sampling in light:dark (LD) conditions, filled circles = sampling after three days in constant darkness (DD). Triangles on the abscissa denote concentrate feeding times. One-way ANOVA for each pattern at all times of year both in LD and DD conditions, $p<0.03$, except in late fall in DD, NS. Two-way ANOVA for each pair of curves (LD, DD) did not show a significant overall effect of lighting at any time of year. (Reprinted from Comparative Biochemistry and Physiology, Part A, Vol. 138, Alila-Johanssson et al., Daily and annual variations of free fatty acid, glycerol and leptin plasma concentrations in goats (Capra hircus) under different photoperiods, pp. 119-131, 2004, with permission from Elsevier.)

There was more fluctuation in glycerol than FFA concentrations. In LD conditions, a constant overall pattern could, however, be detected; the average concentration was relatively high in the evening and at night, and a rapid decline always occurred in the morning (Figure 8). Low levels were usually found until 1400 h. In DD conditions, glycerol concentrations were also observed to decline in the morning in all seasons, but the levels in the afternoon (first part of the sampling period) were high in late spring and summer. Generally, no significant differences were present in overall glycerol concentrations between LD and DD conditions, but in late fall the levels were lower in DD than in LD.

In LD conditions, the overall level of glycerol was lower in summer than in spring and fall. In winter, the level was also lower than in late spring. In DD conditions, similar tendencies were encountered.

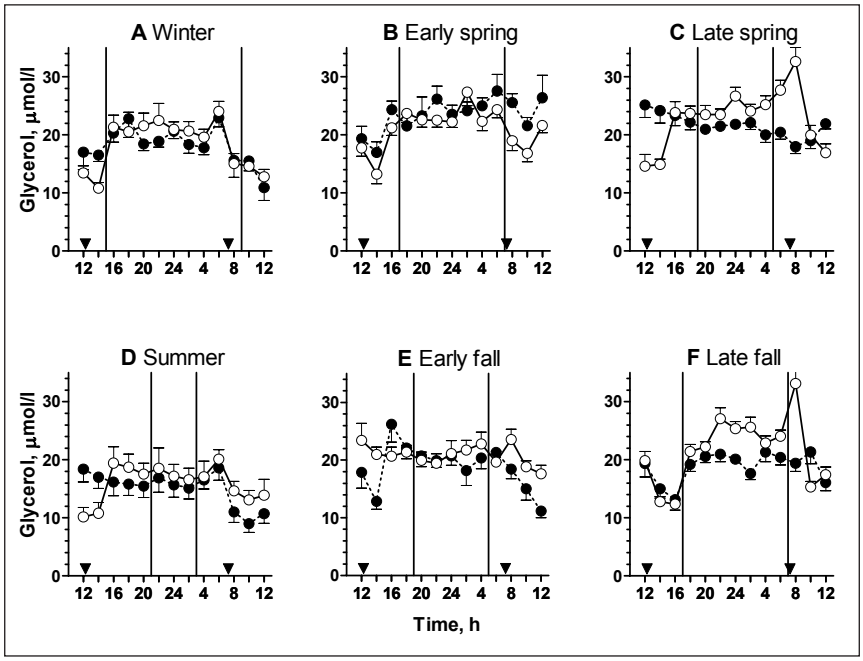

Figure 8 (A-F). Twenty-four-hour patterns of plasma glycerol concentrations in seven goats under various photoperiods during year II (means with SEMs). One-way ANOVA for each pattern at all times of year both in LD and DD conditions, $p<0.02$. Two-way ANOVA for each pair of curves (LD, DD) did not show a significant overall effect of lighting except in late fall: effect of lighting $p<0.05$. (Reprinted from Comparative Biochemistry and Physiology, Part A, Vol. 138, Alila-Johanssson et al., Daily and annual variations of free fatty acid, glycerol and leptin plasma concentrations in goats (Capra hircus) under different photoperiods, pp. 119-131, 2004, with permission from Elsevier.)

FFA and glycerol profiles related to photoperiod

The trough at night and the peak in the morning in the FFA concentration profiles were used as rhythm markers in both LD and DD conditions.

The FFA trough occurred 1.5 – 6 h after the habitual lights-off time (Figure 9). In LD conditions, the interval was shorter in spring and summer than in fall and winter. The FFA trough time paralleled roughly the lights-off time, but it seemed to be delayed about 6 weeks (the interval between samplings) compared with the changes of lighting. The correlation between the FFA trough time and the lights-off time was not significant if the variables of the same time of year were matched. Matching the FFA trough time with the lights-off time of the preceding sampling yielded a highly significant correlation. The FFA trough occurred significantly later in early fall than in winter and early spring. The pattern of annual FFA trough times in DD conditions resembled the pattern in LD conditions, but no significant correlation was found between the FFA trough time and the habitual lights-off time.

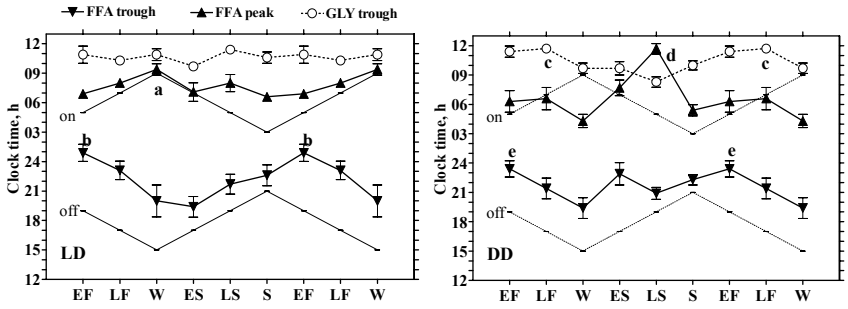

Figure 9. Rhythm marker times (means with SEMs) in the daily profiles of free fatty acid (FFA) and glycerol (GLY) plasma concentrations under different photoperiods during a year. The times were determined visually from individual profiles of seven goats without smoothing. W = winter, ES = early spring, LS = late spring, S = summer, EF = early fall, LF = late fall. EF, LF and W are shown twice to clarify the changes. FFA trough = time of the lowest value of the day, FFA peak = time of the highest value between midnight and noon, GLY trough = time of the lowest value between midnight and noon. LD = sampling in prevailing lighting conditions; lights-off and lights-on times are shown by thin continuous lines without symbols. DD = sampling after three days in continuous darkness: the habitual lights-off and lights-on times are shown by thin dotted lines without symbols. One-way ANOVAs for the annual patterns in LD: FFA trough $p<0.05$, FFA peak $p<0.05$, GLY trough NS; in DD: FFA trough $p<0.05$, FFA peak $p<0.0001$, GLY trough $p<0.05$. a=different from S, b=different from W and ES, c=different from LS, d=different from all other values of FFA peak in DD, e=different from W, $p<0.05$ (Tukey's test). Two-way ANOVAs: overall effect of lighting conditions (LD vs. DD), FFA trough NS, FFA peak $p<0.05$, GLY trough NS. (Reprinted from Comparative Biochemistry and Physiology, Part A, Vol. 138, Alila-Johanssson et al., Daily and annual variations of free fatty acid, glycerol and leptin plasma concentrations in goats (Capra hircus) under different photoperiods, pp. 119-131, 2004, with permission from Elsevier.)

The FFA peak time in LD conditions almost coincided with the lights-on time in late fall, winter and early spring, while under the longer photoperiods (late spring, summer and early fall) the highest FFA concentrations were found 2 – 3.5 h after the lights-on time. The correlation between the FFA peak time and the lights-on time did not reach significance. The peak time occurred significantly later in winter than in early fall. In constant darkness, the annual pattern of FFA peak time was disrupted, mostly due to a marked advance in winter and a delay in late spring (Figure 9).

The morning trough served as a rhythm marker for glycerol concentration profiles. In LD conditions, it occurred constantly at 1000 – 1100 h, and no significant variation according to photoperiod or time of year was observed. In DD conditions, the glycerol trough times were more variable, but were not related to the habitual photoperiod.

The changes of the rhythm marker times seemed to be quite random after the goats had been in constant darkness for 3 days. In most cases, the average shifts were non-significant, but occasional advances or delays of

several hours occurred. No association was present among the shifts of different parameters, nor was any clear trend seen in different seasons.

FFA and glycerol concentrations in different seasons

The daily mean levels of FFA tended to be lower in summer and fall than in winter and spring. The differences were clear in LD conditions, while in DD conditions the annual pattern was more variable.

The annual variations of the daily mean values of glycerol were similar in LD and DD conditions (Figure 10). The lowest levels were found in summer, but also in winter the glycerol levels were lower than in spring.

The daily mean of the ratios of FFA to glycerol concentration in both LD and DD conditions was highest in winter and lowest in late fall, and it seemed to decline through spring, summer and early fall.

During both experimental years, in both LD and DD conditions, the daily mean levels of leptin tended to be low in early fall and high in winter. The differences among the seasons were rather small, and only during the first year in DD conditions was the daily mean level in early fall significantly lower than in winter.

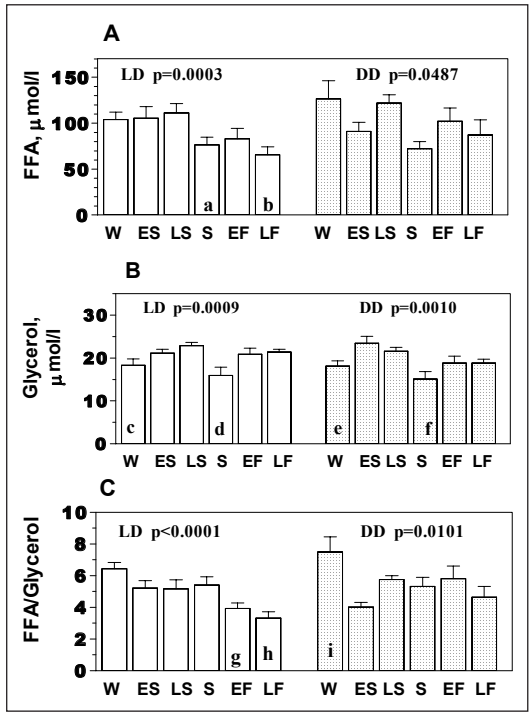

Figure 10. Daily mean levels of free fatty acids (A) and glycerol (B), and FFA/glycerol ratio (C) in seven goats under various photoperiods during year II (means of individual mean levels with SEMs).W = winter, ES = early spring, LS = late spring, S = summer, EF = early fall, LF = late fall. LD = light:dark conditions, DD = after three days in continuous darkness. Results of one-way ANOVAs are shown in the figure. a = different from LS, b = different from W, ES and LS, c = different from LS, d = different from ES, LS, EF and LF, e = different from ES, f = different from ES and LS, g = different from W, h = different from W, ES, LS and S, i = different from ES and LF, $p<0.05$ (Tukey's test). Two-way ANOVAs, overall effects of lighting (LD vs. DD): NS for each variable. Bonferroni's test for selected pairs of values: no significant differences between LD and DD at any time of the year. (Reprinted from Comparative Biochemistry and Physiology, Part A, Vol. 138, Alila-Johanssson et al., Daily and annual variations of free fatty acid, glycerol and leptin plasma concentrations in goats (Capra hircus) under different photoperiods, pp. 119-131, 2004, with permission from Elsevier.)

REPRODUCTIVE STATUS OF ANIMALS AND MEASURED VARIABLES

The sampling days in year I and II fell randomly on different phases of the oestrous cycles of the animals. No significant differences were detected in the daily mean levels of FFA, glycerol or leptin between luteal and non-luteal animals, nor were the correlations significant between progesterone levels and the daily mean values of the metabolites or leptin (both parametric and rank order calculations performed).

SUMMARY OF THE MAIN RESULTS OF MEASUREMENTS

Table 2. Phase of the daily rhythm and seasonal variation of blood levels of melatonin, cortisol, leptin, FFA and glycerol in LD (left column) and DD (1 and/or 3 days, right column)

PHASE OF THE DAILY RHYTHM		
	LD	**LD -> DD**
Melatonin	According to lighting (mainly light offset): High levels during darkness	1 day: advances of rise times except in winter 3 days: advances of rise and decline times
Cortisol	No distinct rhythm	1 or 3 days: no changes*
Leptin	No distinct rhythm	1 day: no changes (3 days: not measured)
FFA	According to lighting (mainly light onset): peak levels around light onset	3 days: rise time advances in fall and winter
Glycerol	According to feeding times: trough levels after the concentrate meals	3 days: no changes
SEASONAL VARIATION		
	LD	**LD -> DD**
Melatonin	Peak duration: according to scotoperiod except in winter Peak level: no variation Mean level: high in winter, low in summer	1 day: peak duration increases in summer and early fall, mean level increases in summer 3 days: peak duration and mean level increase in summer
Cortisol	High levels in winter Low levels in spring and summer	1 or 3 days: no changes in overall levels*
Leptin	No distinct variation	1 or 3 days: no distinct changes
FFA	High levels in winter and spring Low levels in summer and fall	3 days: no changes in overall levels
Glycerol	High levels in spring and fall Low levels in winter and summer	3 days: no changes in overall levels
FFA/glycerol	High values in winter Low values in fall	3 days: no changes in overall values

* Measurements of cortisol after 3 days in DD are unpublished

RELATIONSHIPS BETWEEN MELATONIN AND FFA CONCENTRATIONS (IV)

The average concentration profiles suggested that there was some association between low FFA and high melatonin levels. In addition, the decline of melatonin in the morning seemed to precede the FFA peak levels in most cases.

Phase marker times

Under LD conditions, the melatonin onset was very close to the lights-off time in all seasons. Melatonin offset times were mostly around the lights-on time except in winter; under the 18-h darkness, the melatonin concentrations decreased to the threshold level more than 4 h before lights-on. Thus, the duration of high melatonin levels reflected the length of the dark period in all other seasons except winter. After 3 days in DD, both melatonin onset and offset tended to advance. The mean advance of melatonin offset was significant under the longest scotoperiods in late fall, winter and early spring (Figure 11).

Under LD conditions, the FFA half-rise time preceded the lights-on time under the shorter photoperiods in late fall, winter and early spring; while under the longer photoperiods in late spring, summer and early fall, the FFA half-rise time occurred around the lights-on time. After 3 days in DD, the FFA half-rise time advanced in all seasons. The most marked advances occurred in late fall and winter (3 – 4 h), and a significant advance also occurred in early fall.

Phase differences between FFA half-rise time and melatonin phase marker times

The phase difference between melatonin onset and FFA half-rise time varied in LD conditions from about 6 h in summer to about 16 h in winter, roughly paralleling the duration of darkness. Under DD conditions, the interindividual variation in the phase differences increased and the variation among the seasons decreased. Although the mean interval from melatonin onset to FFA half-rise time decreased in winter and increased in summer, the difference of the intervals remained significant between winter and summer. As in winter, the phase difference from melatonin onset to FFA half-rise time decreased significantly in DD also in early and late fall.

Melatonin offset and FFA half-rise times were rather similar in both LD conditions and after 3 days in DD. On average, the FFA half-rise time occurred slightly before the melatonin offset. Under LD conditions, the winter pattern was exceptional due to the relatively long interval from melatonin offset to FFA half-rise time (about 2 h). After 3 days in DD, this exception disappeared, and there were no significant differences in the intervals between melatonin offset and FFA half-rise time among the seasons.

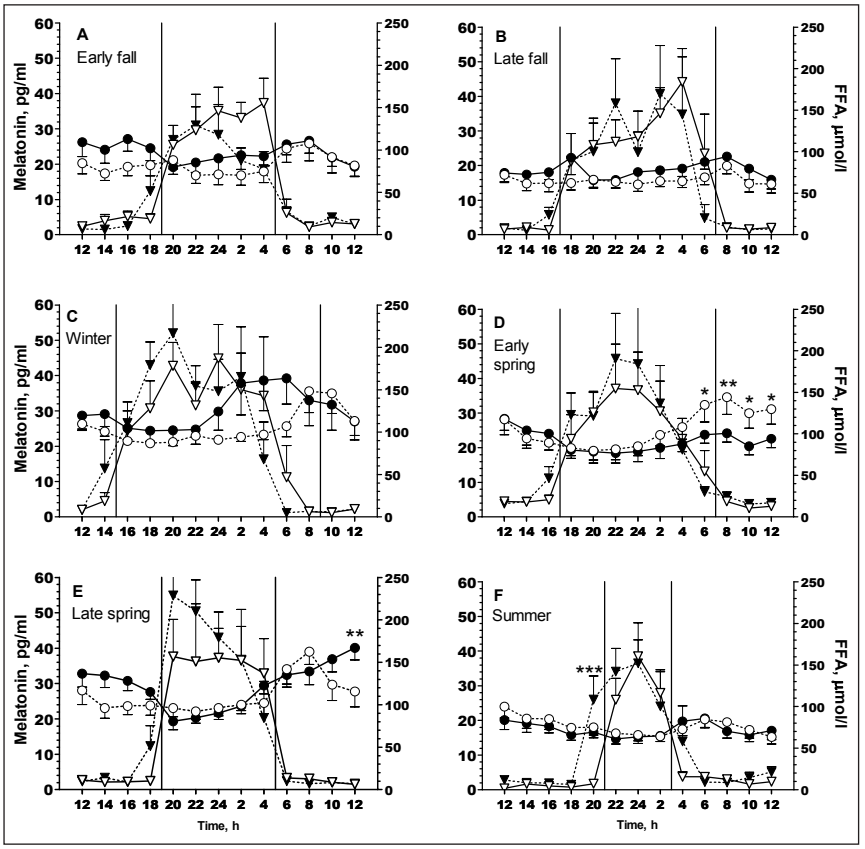

Figure 11 (A-F). Twenty-four-hour patterns of serum melatonin (triangles) and plasma free fatty acid (FFA) (circles) concentrations in seven goats under various photoperiods during a year (means with SEMs). The animals were kept in artificial lighting simulating annual natural photoperiods. The habitual dark period is shown as an interval between the vertical lines in each figure. Blood samples were collected at 2 h intervals from 1200 to 1200 h after the respective lighting regime had been maintained at least for 24 days. Open symbols = sampling in light:dark (LD) conditions, filled symbols = sampling after three days in constant darkness (DD). One-way ANOVA for each pattern of melatonin in all seasons both in LD and DD conditions: effect of time, $p<0.0001$; FFA: $p<0.03$ for all curves. Two-way ANOVAs for each pair of melatonin curves (LD, DD) indicated a significant effect of lighting on the overall level only in summer ($p<0.02$) and for FFA curves in none of the seasons. Significant interaction effects lighting x time were found for melatonin in late spring ($p<0.02$) and in summer ($p<0.002$), and for FFA in early spring ($p<0.01$) and in late spring ($p<0.001$). Bonferroni's test: * different from the corresponding value in the other lighting, $p<0.05$; ** $p<0.01$; *** $p<0.001$. (Reprinted from Chronobiology International, Vol. 23(3), Alila-Johanssson et al., The daily rhythm of melatonin and free fatty acids in goats under varying photoperiods and constant darkness, pp. 565-581, 2006, with permission from the Taylor & Francis Group.)

Correlations among rhythm parameters

A very significant positive correlation was found between melatonin onset and lights-off time under LD conditions. Melatonin offset did not correlate

significantly with the lighting transitions. After 3 days in DD, the significant correlation persisted between melatonin onset and habitual lighting conditions.

Under LD conditions, the FFA rhythm parameters correlated significantly or almost significantly with both lighting transitions and melatonin onset. Under DD conditions, the FFA rhythm parameters did not correlate significantly with habitual lighting transitions or melatonin onset.

Melatonin offset was not related to the FFA rhythm parameters in LD conditions, while a positive correlation between melatonin offset and FFA half-rise time was found in DD conditions.

DISCUSSION

DAILY AND ANNUAL PATTERNS OF SERUM MELATONIN

Melatonin profiles in LD and DD conditions

The duration of melatonin secretion of the goats was in line with the length of the dark phase in all LD conditions, except winter. The high levels reached within an hour after lights off were usually maintained until the lights were turned on. Under the 18-h scotoperiod in winter, however, the duration in all goats was significantly shorter (mean 14 h), suggesting the existence of an intrinsic maximum duration of secretion. This type of melatonin pattern has also been observed in other breeds of goats (Maeda et al., 1984; Kanematsu et al., 1989; Deveson et al., 1990) and in, for example, sheep (Reiter, 1993). The results showed that although the overt melatonin signal carried information about the length and timing of the prevailing dark period, it did not differentiate between increasing and decreasing photoperiods in spring and fall.

To study the intrinsic pattern of melatonin secretion under the different photoperiods of the year, we determined the patterns also in constant darkness. The immediate melatonin profiles in the DD conditions, which were considered to reflect the intrinsic melatonin pattern in the prevailing habitual lighting conditions, showed that: 1) the duration of the endogenous melatonin peak was shorter in late spring and summer than in other seasons, 2) in equal habitual photoperiods in late spring and early fall, the duration of the melatonin peak increased in the latter but not in the former and 3) in all seasons, except in winter, the melatonin onset advanced in DD.

Role of intrinsic clock and light transitions in the generation of melatonin synthesis

The hypothalamic body clock generates the endogenous rhythm of melatonin. The pacemaker in the SCN is entrained to the lighting transitions and it can be adjusted according to varying photoperiods. The concept of separate evening and morning oscillators (Pittendrigh and Daan, 1976; Illnerova and Vanecek, 1982; Elliott and Tamarkin, 1994) has had novel interpretations based on the expression of several clock genes in the SCN (Nusslein-Hildesheim et al., 2000; Daan et al., 2001; Hofman, 2004) and in terms of transmitter release (Shinohara et al., 1995; Noguchi et al., 2004) or

electrical activity (Jagota et al., 2000) in the SCN slices. The result of Study I showed that when the scotoperiod decreased from 10 to 6 h or increased from 14 to 18 h, no further changes occurred in the duration of the endogenous melatonin profiles. The maximum difference between the duration of unmasked melatonin signals was 3-4 h, suggesting that photoperiodic variation of this length is sufficient to entrain the circannual rhythms of goats.

The melatonin rhythm in Finnish landrace goats is most probably dominated by the evening oscillator; in all animals in all seasons, the melatonin levels increased immediately after the light offset, whereas more interindividual variation existed in the timing of melatonin decline, and in winter the high melatonin levels were not maintained until light onset in any animal. Melatonin offset was adjusted by dawn only if the dark period was 14 h or shorter. It seems that the goat has effective intra- or extrapineal feedback mechanisms that enable melatonin synthesis to be arrested on the basis of its start time independently of the continuing darkness.

Difference in melatonin profiles between spring and fall

The results showed a tendency of the melatonin profile to increase symmetrically in DD conditions, more in early fall than in late spring, irrespective of the equal habitual photoperiods maintained for four weeks. This spring-fall difference might reflect two phases of an endogenous circannual clock mechanism or it could be a sign of long-term photoperiodic history. Other seasonal cues could also have contributed to this finding because the animals spent two months outdoors in summer.

Length of the free-running period

In DD conditions, the melatonin onset time advanced consistently when the habitual dark period was 14 h or shorter. This result suggests that there is an endogenous period of less than 24 h in the oscillator regulating the melatonin onset time in goats. The interpretation is corroborated by the finding that even in winter the melatonin level always rose immediately after lights-off, while it declined well before lights-on. We have not come across any reports on caprine free-running periods. Most breeds of sheep have a period longer than 24 h (Ravault et al., 1989; Earl et al., 1990a and b; Matthews et al., 1992), except Soay rams, whose free-running period has been reported to be shorter than 24 h (Kumar and Lincoln, 1995). The Finnish landrace goat may belong to the mammalian species having a short free-running period and consequently a dominant "evening oscillator" (cf. page 15) for the adjustment of the melatonin rhythm.

DAILY AND ANNUAL PATTERNS OF SERUM CORTISOL

Reports of circadian variation of cortisol levels in ruminants somewhat contradictory

In agreement with previous studies in this breed of goats, we found no significant daily rhythmicity in serum cortisol levels (Eriksson and Teräväinen, 1989).

Several reports exist of ruminants in which no clear variation of cortisol levels was detected (Basset, 1974; Paape et al., 1974; Hudson et al., 1975; Barrell and Lapwood, 1978; Lincoln et al., 1982; Bubenik et al., 1983; Simonetta et al., 1991; Ingram et al., 1999), whereas in some other studies a significant or more constant daily variation of cortisol has been observed (MacAdam and Eberhart, 1972; McNatty et al., 1972; Wagner and Oxenreider, 1972; Holley et al., 1975; Fulkerson and Tang, 1979; Fulkerson et al., 1980; Kennaway et al., 1981; Thun et al., 1981; McMillen et al., 1987; Lefcourt et al., 1993; Lyimo et al., 2000). In the latter studies, the samples have usually been collected frequently or the measured values have been pooled. On the basis of the above results, ruminants seem to have a weak intrinsic daily cortisol rhythm that is easily masked by external factors. The cortisol profiles of our goats, which were allowed to eat *ad libitum,* were rather monotonous compared with the profile for goats fed three times a day (Eriksson and Teräväinen, 1989). Similarly, daily variation in plasma cortisol concentrations was found in pregnant ewes fed once a day, but not in ewes fed at frequent intervals throughout the day (Simonetta et al., 1991).

In addition to asynchrony of feeding, rumination and the stress related to experimentation may be factors underlying the lack of rhythmic cortisol secretion. Hudson and co-workers (1975) have shown in cattle that rumination prevents the animals from entering deep sleep. Our goats were also at least momentarily prevented from sleeping by the blood sampling occurring every 2 h. Light or fragmented sleep is, however, not necessarily the sole reason for the lack of cortisol rhythm. In humans waking throughout the night, cortisol rhythm does not disappear (Wehr, 1998).

As to the stress response related to the experimentation, we believe that this factor does not provide a probable explanation for the weak cortisol rhythm because our animals were cooperative and accustomed to frequent handling and various kinds of experimental procedures.

Effect of light on short-term regulation of cortisol levels

Although in day-active animals the morning rise of cortisol is endogenous, in humans the morning rise of cortisol can be enhanced by bright light after awakening or sleep deprivation (Scheer and Buijs, 1999; Leproult et al., 2001). Similarly in pigs and male Creole goats, cortisol levels may increase immediately after exposure to supplementary artificial light in the morn-

ing after sunrise or after an abrupt exposure to sunlight in the middle of the day (Sergent et al., 1985; Andersson et al., 2000). Whether these phenomena are some kind of stress response or more specific effects of light is not known. In our study light had no impact in the short-term regulation of cortisol levels, but a long-term regulation by light seems probable because the levels varied under different photoperiods.

Highest cortisol levels occur in winter and the lowest in summer

The high cortisol level in winter decreased with increasing photoperiod in spring and the low summer levels increased in fall. In spring and fall in equally long photoperiods, the cortisol levels were not the same. This finding indicates that not only the photoperiod but also the direction of the change affects the cortisol levels.

Our finding of seasonal changes in the cortisol levels is in contrast with the results of the only study that we have found on this topic in the goat. Howland and co-workers (1985) found no monthly variations of the cortisol level in the pooled samples collected from pygmy goats each month of the year. On the other hand, seasonal and photoperiodic variation of cortisol levels has been found in most studies in other ruminant species (Bubenik et al., 1975, 1977, 1983; Leining et al., 1980; Kennaway et al., 1981; Lincoln et al., 1982; Brinklow and Forbes, 1984; Bubenik and Brown, 1989; Monfort et al., 1993; Feher et al., 1994; Ingram et al., 1999). The discrepancy between the study of Howland and co-workers (1985) and our investigation might be explained by differences of these studies with respect to the strain and sex of animal, and experimental procedures and conditions.

Mechanism underlying seasonal changes in cortisol levels

The most obvious interpretation of the higher cortisol levels in winter than in summer is that it resulted from the marked difference (12 h) between the photoperiods in winter and summer at a latitude of 60°N since our goats stayed indoors in constant conditions, except for lighting, during 10 months of the year and received high-quality food *ad libitum*. However, it is possible that the photoperiod as such does not regulate cortisol secretion. Instead, through the hypothalamic feedback regulatory system, the photoperiod may act as a trigger of reproductive functions involving cortisol secretion. This view is supported by the finding that in lambs pinealectomy prevented the photoperiod-induced changes in cortisol levels as it prevents changes in prolactin and testosterone secretion (Brinklow and Forbes, 1984).

Although seasonal changes of cortisol levels might be related to alterations in the secretion of reproductive hormones, there is no evidence that elevated cortisol levels are connected to high testosterone levels *per se* during the reproductively active period of male goats (Howland et al., 1985),

sheep (Lincoln et al., 1982) or white-tailed deer (Bubenik et al., 1983).

The results of Study II indicate that the daily rhythm of serum cortisol levels in our female goats is mainly entrained by external conditions, the strongest Zeitgeber being the feeding schedule. On the other hand, the duration of the photoperiod and the direction of its change during a year significantly influenced the overall cortisol levels. This finding is suggested to be associated with the reproductive functions of the animals.

DAILY AND ANNUAL PATTERNS OF SERUM LEPTIN

No daily rhythm was present in leptin levels in our goats, and thus, the release of leptin seemed to have no role in the daily changes of lipid metabolism. In agreement with our findings, some studies have described no daily rhythmicity of leptin levels in sheep (Blache et al., 2000; Tokuda et al., 2000; Marie et al., 2001; Daniel et al., 2002).

In many species, e.g. sheep and cows, plasma leptin levels have been found to correlate with adiposity (Blache et al., 2000, Delavaud et al., 2000, Ehrhardt et al., 2000).

Thus, the small seasonal change of leptin (decrease in early fall) found in this study can possibly be explained by the small decrease in body mass. However, the relative constancy of our leptin levels does not rule out the possibility of light-dependent changes; the method of measurement (the "multispecies" antibody) may have been insufficiently sensitive to detect small changes in leptin levels in the goat (Blache et al., 2000; Delavaud et al., 2000; Ehrhardt et al., 2000).

In ruminants, inconsistent daily patterns have been described for cortisol, insulin, glucagon, growth hormone, prolactin, thyroid hormones and catecholamines (Hart, 1973; Bassett, 1974; Barrell and Lapwood, 1978; Fulkerson et al., 1980; Kennaway et al., 1981; Vasilatos and Wangsness, 1981; Lincoln et al., 1982; Bubenik et al., 1983; Brinklow and Forbes, 1984; Blum et al., 1985, 2000; Sutton et al., 1988; Bitman et al., 1990; Marie et al., 2001). The variations, if any, were connected to the feeding or lighting conditions, depending on the experimental design. Light-dependent variations of several hormones can adjust the light-dependent component in the daily rhythm of lipid metabolism. We seem to have excluded the direct effects of cortisol (II) and leptin (III) concentrations.

In ruminants, the secretion of several metabolic hormones is more related to variations of photoperiod than to variations of circadian rhythms, but the causal relationships among photoperiod, body weight and hormonal levels are not yet fully understood (reviewed by e.g. Lincoln and Richardson, 1998; Rhind et al., 2002). According to our findings lipid metabolism can be affected by photoperiod without concomitant changes in body mass or leptin levels.

Daily variations related to the photoperiod occurred in the FFA levels: they decreased at night and increased in the morning. Glycerol levels were associated with meal times. Annual changes were detected in overall FFA and glycerol levels; FFA levels were low in summer and fall, and high in winter and spring, whereas glycerol levels in summer and winter were lower than in spring and fall.

Morning rise of plasma FFA

The increase of plasma FFA concentration in the early morning is in line with the findings of earlier studies in ruminants (Blum et al., 1985, 2000; Marie et al., 2001). In these investigations, the increase has usually been suggested to be due to mobilization of the lipid reservoir from adipose tissue in response to reduced food consumption during the night.

Since the rise of FFA in the morning was not accompanied by a rise in glycerol concentration, it is probably unrelated to increased lipolysis, instead reflecting the intrinsic circadian rhythm of lipid metabolism, adjusted by external factors, such as lighting conditions and times of food rationing or intake of main meals. Recent evidence suggests that hepatic glycerol uptake is more efficient than hepatic FFA uptake and that muscle and adipose tissues are capable of taking up glycerol from the circulation (Koutsari and Jensen, 2006). That the daily variations of FFA levels were a manifestation of an intrinsic circadian rhythm was supported by the finding that irregularities occurred in the rhythm after 3 days in constant darkness. However, light as the sole entrainer of this rhythm could not be proved because the patterns of hay intake and/or rumination were not measured. According to our observations, however, the concentrate meals rationed out at constant times throughout the experiment were immediately consumed by the goats, within about 5 min. This observation suggests that the circadian rhythm of FFA is at least partly dissociated from the times of high-energy meals and regulated by light — directly or indirectly. This suggestion is supported by the finding that the SCN is neurally connected to white adipose tissue through the sympathetic nervous system (Bartness et al., 2001).

Daily rhythm of glycerol

Glycerol rhythm was found to be dictated by the concentrate meals, and the daily variation remained constant throughout the year and in constant darkness. The finding that the glycerol levels were lower during eating concentrate meals (0700 and 1200 h) than during the period of hay intake and rumination suggests a high rate of lipogenesis in relation to lipolysis. In summary, the glycerol measurements support the view that in goats lipid metabolism is directly regulated by feeding.

Seasonal changes in lipids and body mass in relation to each other and to photoperiod

Seasonal changes were observed in overall FFA and glycerol levels. The lower plasma FFA concentrations in summer and fall suggest increased lipogenesis and the higher concentrations in winter and spring decreased lipogenesis. On the other hand, the higher plasma glycerol concentrations in early and late spring suggest increased lipolysis and the lower levels in summer increased lipogenic activity. The FFA/glycerol ratio was at its lowest in fall, probably reflecting the time of the most active lipogenesis.

The body mass of the goats did not change much (except in early fall when it was at its lowest 4 weeks after the grazing period), suggesting a constant balance between food consumption and energy requirement. In early fall, the loss in mass, based on observations over several years, is likely due to increased physical activity in the pasture instead of receiving food accompanied by less exercise in indoor pens.

The increased body mass and the decreased FFA concentration and FFA/glycerol ratio occurring in early fall indicate higher lipogenesis at this time of the year. At other times of the year, the variations in metabolite concentrations did not correlate with the changes in the body mass. Thus, lipid metabolism seems to be more sensitive to photoperiod than body mass. This view is in line with the findings of Lincoln and co-workers (2001), who showed that it took several weeks until food intake and body mass started to decrease in sheep moved from long to short photoperiods.

Light, suprachiasmatic nucleus (SCN) and autonomic nervous system in regulation of lipid metabolism

Light can regulate lipid metabolism from the hypothalamus directly through the autonomic nervous system (Bartness et al., 2001; la Fleur, 2003). An increased sympathetic tone in the morning (entrained to the habitual LD conditions) could explain the rise of FFA levels. A decrease of FFA levels might then result from feeding-dependent hormonal changes (e.g. an increase of insulin release). Bartness and co-workers (2002) suggested that under short photoperiods increased lipolysis could reflect increased sympathetic drive to the adipose tissue and that increased secretion of melatonin affects the SCN and enhances sympathetic tone in Siberian hamsters. In our goats, the secretion of melatonin in winter was more than twofold that in summer (I).

The results of Study III indicate that lighting conditions participate in the regulation of the daily pattern and annual variations of lipid metabolism in the goat. A novel finding was that the daily FFA rhythm was dependent on the LD conditions. The high sensitivity of lipid metabolism to the photoperiod shows the special importance of light for adaptive mechanisms, even in domestic animals without any changes in food consumption.

RELATIONSHIP BETWEEN SERUM MELATONIN AND PLASMA FREE FATTY ACIDS

An overall parallelism was found between melatonin and FFA rhythms. There was, however, one exception; in winter, in LD conditions, the morning rise in FFA levels coincided with lights-on and not with the declining phase of melatonin, whereas, in constant darkness, the FFA peak advanced several hours and coincided with the declining phase of melatonin. The parallelism of melatonin and FFA rhythms observed in this study during the natural 24 h photoperiod may have arisen through several different mechanisms: 1) the FFA levels may be assessed by direct effects of light, independently of melatonin or clock mechanisms, through the retinohypothalamic pathways and from the hypothalamus to the hormonal effector pathways and/or autonomic nervous system, 2) the melatonin and FFA rhythms may be regulated by a common endogenous, light-controlled oscillator or by parallel interconnected oscillatory systems or 3) the FFA rhythm may be hierarchically submissive to melatonin.

Absence of direct effect of light on FFA levels

Light has been shown to have a direct suppressing effect on melatonin synthesis in many species, including the goat (Maeda et al., 1984; Deveson et al., 1990). In concert with these findings, the FFA peak in this study occurred around the lights-on time in most cases, supporting an alerting lipolytic effect of light, as demonstrated in humans (Campbell et al., 1995; Perrin et al., 2004). However, strong evidence against the direct effect of light was provided by our finding that regular daily variation of FFA levels was maintained in constant darkness.

Melatonin and evening oscillator (cf. page 15)

The melatonin levels in our goats increased immediately after the light offset in all seasons of the year. Melatonin rhythm is therefore most probably dominated by the evening oscillator. The timing of the melatonin decline varied interindividually. In winter, the high melatonin levels were not maintained throughout the dark period in any animal, and therefore, the melatonin signal did not measure exactly the longest 18-h dark period. The beginning of darkness was signalled better than the end. Melatonin offset was adjusted by dawn only if the dark period was 14 h or shorter. Thus, the length of melatonin synthesis in Finnish female Landrace goats depends on its start time independently of the continuing darkness; effective intra- or extrapineal feedback mechanisms that arrest the synthesis must therefore exist.

The role of melatonin and light-dependent mechanisms could not be differentiated in the regulation of the FFA rhythm because the FFA rhythm markers and the melatonin onset were clearly related to the lighting tran-

sitions in LD conditions. However, no significant correlations existed between melatonin onset and FFA rhythm marker times in DD conditions. Thus, melatonin onset, i.e. the rising phase of melatonin (evening oscillator), does not share a common rhythm generating system with the endogenous FFA rhythm.

FFA rhythm and morning oscillator (cf. page 15)

In LD conditions, melatonin offset was not related to FFA rhythm marker times. In DD conditions, it was related to the FFA half-rise time. An explanation for this might be that the FFA rise is controlled by a morning oscillator, which in LD conditions is set by the lights-on time, whereas in DD conditions, some intrinsic setting, e.g. a time schedule in the expression of clock genes in darkness or cues other than light must exist. A Zeitgeber besides light might be the decline of melatonin levels. The results indicate, however, that the mechanisms regulating the melatonin offset and FFA rise are not exactly the same, although their phases coincide in DD conditions.

In DD conditions, a positive correlation was present between the phase shifts of melatonin offset and FFA half-rise time, but no seasonal variation of phase differences between melatonin offset and FFA half-rise time occurred. This finding is in agreement with the view that the FFA rhythm is more closely related to melatonin offset than melatonin onset, supporting the assumption that the FFA rhythm is generated by a hypothalamic oscillatory system, i.e. the components of the morning oscillator have a greater role than the evening oscillator in the generation of the rhythm.

Effect of melatonin on FFA rhythm via the nervous system

One possibility is that the parallelism of the daily rhythms of melatonin and FFA concentrations results from melatonin affecting the lipolysis/lipogenesis balance, either through hypothalamic mechanisms or locally in peripheral tissues. Specific melatonin binding sites have been shown to exist in the suprachiasmatic region of goats (Deveson et al., 1992) and the SCNs have been implicated in the regulation of energy metabolism in several other species (reviewed e.g. by Nagai et al., 1994; Bartness et al., 2001; Buijs et al., 2003; la Fleur, 2003). In Siberian hamsters, the suprachiasmatic neurons connected to white fat have been demonstrated to express melatonin receptor mRNA (Song and Bartness, 2001). Moreover melatonin injections during daytime can decrease FFA levels in cows (Darul and Kruczynska, 2004) and rats (Mazepa et al., 2000); the nature of the mechanism, whether central or peripheral, has not ,however, been elucidated.

Effect of melatonin on FFA rhythm via a peripheral mechanism

Peripheral melatonin receptors in ruminants have not received much research interest. In species other than ruminants, however, the existence of

peripheral melatonin receptors has been demonstrated; the sites include adipose tissue and liver (Acuna-Castroviejo et al., 1994; Le Gouic et al., 1997; Brydon et al., 2001; Poon et al., 2001; Sauer et al., 2001; Zalatan et al., 2001; Naji et al., 2004; Sallinen et al., 2005). Sauer and co-workers (2001) suggested a dual local effect of melatonin on the basis of a study on rat inguinal fat tissue *in situ*; physiological concentrations of melatonin decreased the release of fatty acids in fasted rats and decreased the uptake of fatty acids in fed rats. Furthermore, studies *in vitro* have shown that melatonin has an inhibitory effect on lipid metabolism or fatty acid transport (Ng and Wong, 1986; Blask et al., 1999; Zalatan et al., 2001; Dauchy et al., 2003).

In our goats, FFA levels were low and melatonin levels high at night. This finding supports the view that high melatonin concentrations might have an inhibitory influence on lipolysis and FFA release. However, the role of melatonin in the generation of the overall FFA rhythm is questionable; in winter in LD conditions, the morning rise in FFA levels coincided with lights-on and not with the declining phase of melatonin, whereas in DD conditions in winter, the FFA peak advanced several hours and coincided with the declining phase of melatonin.

In summary, the results of Study IV support the view that the daily rhythm of blood FFA levels is regulated by an intrinsic clock, which in turn is controlled by light, especially by dawn. The close relationships between the daily variations of melatonin and FFA levels result from both variables being controlled by light-dependent mechanisms.

CONCLUSIONS

On the basis of these results, the following conclusions can be made:

I In addition to the light-adjusted overt melatonin rhythm, the endogenous melatonin secretion can be modulated by circannual clock mechanisms and/or long-term photoperiodic history. The duration of melatonin secretion in goats closely follows the length of the dark phase, except in winter, when the duration is significantly shorter. In DD, the goats display two types of endogenous melatonin patterns: a "winter pattern" in winter, early spring, early fall and late fall, and a "summer pattern" in late spring and summer.

II Serum cortisol levels have no significant daily rhythm at any time of the year, nor are there any differences in the profiles between LD and DD. In winter, however, the overall concentrations were higher than in any other season. The results suggest that any/the circadian variation of cortisol secretion is masked by external factors, probably by the feeding schedule, and that the seasonal variation in the overall cortisol levels is likely to be related to the changes in photoperiod.

III The lipid metabolism of goats is regulated by light; no significant contribution of leptin levels could be shown. Concentrations of plasma FFA and glycerol exhibit significant daily and seasonal variations. The photoperiod was found to trigger a nocturnal fall and morning rise of FFA levels, while low levels of glycerol were associated with the concentrate meal times.

IV The daily rhythm of FFA is generated by an endogenous oscillator, primarily adjusted by dawn, whereas the melatonin rhythm is regulated by an oscillator primarily adjusted by dusk. There was an overall parallelism between the two rhythms, with one significant exception: in winter in LD conditions, the morning rise of FFA levels coincided with lights-on and not with the declining phase of melatonin, whereas in DD conditions, the FFA peak advanced several hours and coincided with the declining phase of melatonin. The results do not rule out a possible effect of melatonin on the daily FFA profiles, but they do show that melatonin secretion alone does not sufficiently explain the patterns.

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